UNIVERSITY OF ALBERTA

THE ROLE OF LACCASE FROM *CORIOLOPSIS GALLICA* IN POLYCYCLIC AROMATIC HYDROCARBON METABOLISM

BY

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Abstract

In a search for new strains with enhanced metabolic activities, *Coriolopsis gallica* UAMH 8260 was studied for polycyclic aromatic hydrocarbon degradation. A medium containing 3% Kellogg's Bran Flakes in 60 mM phosphate buffer, pH 6, supported high levels of laccase production, 13 units ml^{-1}, by *C. gallica*. In bran flakes medium *C. gallica* demonstrated 9.4% mineralization of ^{14}C-phenanthrene over 42 d. Bran flakes medium amended with the laccase mediators 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) and 1-hydroxybenzotriazole, to a 1 mM final concentration enhanced mineralization to 17.5 %. Laccase was purified from the spent medium of *C. gallica* grown in submerged culture in 8L bran flakes medium for 9 d in a 14 L stirred tank fermentor. The culture exhibited a laccase activity of 15 units ml^{-1}, among the highest laccase levels described for a wild-type fungus. The purified enzyme exhibited characteristics typical of a white rot fungal laccase. Metabolism of polycyclic aromatic hydrocarbons was compared using the native laccase and enzyme forms chemically modified by polyethylene glycolation and methylation. Results showed that chemically modified laccase was able to metabolize an increased number of polycyclic aromatic hydrocarbons and the first order kinetic constant increased for some substrates.

The University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Role of Laccase from *Coriolopsis gallica* in Polycyclic Aromatic Hydrocarbon Metabolism submitted by Heather A. Vandertol-Vanier in partial fulfillment of the requirements for the degree of Master of Science. in microbiology and biotechnology.

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1. Introduction.

1.1 Bioremediation.

Bioremediation is a process that removes xenobiotic compounds from the biosphere. This process employs microorganisms or plants to remove the contaminating organic compounds by metabolizing them to carbon dioxide and into biomass. This method enhances biodegradation processes that already exist in nature (Alexander, 1994). The purpose of bioremediation is to degrade pollutants to undetectable concentrations or to concentrations that are below the limits established by regulatory agencies. Bioremediation has been used to degrade xenobiotic contaminants in soils, ground water, waste water, sludges, industrial waste and gases (Alexander, 1994).

1.2 History of medium composition.

The research in this thesis originated from studies at the Institute of Biotechnology UNAM in Mexico and the Department of Biological Sciences at the University of Alberta. The objective of these projects was to study white rot fungi (WRF) and their biotechnological roles in processes such as dye decolorization and bioremediation of polycyclic aromatic hydrocarbon (PAH) contaminated soils.

Several WRF from the University of Alberta Mold Herbarium (UAMH) were found to be active in polychlorinated biphenyl (PCB) congener metabolism and mineralization (Beaudette et al., 1998). It was thought that these fungi might be able to extend their degradative ability to PAHs. It had been shown that purified ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase were able to metabolize a number of PAHs *in vitro* (Bohmer et al., 1998, Bumpus, 1989; Collins et al., 1996; Hammel et al., 1991; Johannes et al., 1996; Majcherczyk et al., 1998; Vazquez-Duhalt et al., 1993). White rot fungi have been surveyed for enzymes involved in lignin degradation. These surveys have demonstrated that ligninolytic enzymes are widespread throughout this group. There is considerable variation in the type of ligninolytic enzyme and the levels of

enzyme produced by various strains of the same species. Enzyme production can depend on culture conditions and medium composition. Before this study could proceed, a medium was needed that enhanced production of laccase to high levels. Numerous compositions and modifications of media have been described in the literature that have enhanced lignin peroxidase levels in *Bjerkandera* sp. strain BOS55 and strains of *Trametes versicolor* and *Phanerochaete chrysosporium* (Kaal et al., 1993; Mester et al., 1996; Collins et al., 1997). However, these media were unable to enhance enzyme production in *C. gallica*. Even 2,5-xylidine, a commonly used efficient laccase inducer, could not enhance laccase production in *Coriolopsis gallica* as it had for strains of *T. versicolor* and *Pleurotus ostreatus* (Pickard et al., 1999). Solid-state fermentation with whole oats has been used to grow *C. gallica* and was shown to enhance laccase production (Rodriguez et al., 1999). It appeared that a natural lignin source enhanced laccase production. Similar carbon sources were investigated for their ability to enhance laccase production in submerged cultures of *C. gallica*. Further studies were needed to determine the best conditions for laccase production by *C. gallica*. As well, sufficient quantities of laccase were needed for purification, characterization and investigation of their role in PAH metabolism.

1.3 History of PAH metabolism.

White rot fungi can completely mineralize some PAHs, indicating that complete oxidation of PAHs occurs. However, there are few examples of *in vitro* oxidation of PAHs by culture supernatants and purified enzymes. The oxidation of anthracene and pyrene by lignin and manganese peroxidases from *P. chrysosporium* and oxidation of many PAHs by the laccases of *T. versicolor* have been reported (Bumpus, 1989; Bohmer et al., 1998; Collins et al. 1996; Hammel et al., 1991; Johannes et al., 1996; Vazquez-Duhalt et al., 1993). In a study by Pickard et al. (1999), it was shown that previously uncharacterized fungal strains could metabolize selected PAHs *in vivo*. *C. gallica* was one of the strains studied and was found to degrade several PAHs. Anthracene concentration decreased

by up to 90%, pyrene, up to 20% and phenanthrene, up to 40%. Therefore, further studies were required in this area. It was also observed that partially purified laccases were able to metabolize PAHs *in vitro* and the accelerative ability of mediators in the oxidation of PAHs was demonstrated for anthracene. Further studies were needed to determine the extent of PAH metabolizing ability of *C. gallica*.

1.4 Research objectives.

The research objectives of this thesis were:

1. To develop a medium that would produce high levels of laccase by *C. gallica* to be followed by large-scale production, purification and characterization of laccase.
2. To assess the ability of the fungus to utilize PAHs by determining its ability to mineralize PAHs *in vivo* and to metabolize PAHs *in vitro* using purified laccase.
3. To chemically modify the laccase produced by *C. gallica* and to compare the ability of these modified laccases to metabolize PAHs.

2. Literature Review.

2.1 White rot fungi.

A variety of microorganisms are involved in wood decomposition, however the dominant decomposers are fungi. Different processes are used to degrade wood because it is composed of differing complex components. Three types of filamentous fungi are able to utilize wood that have a preference for one or more of the wood polymers. To categorize these forms of decay, the color and texture of the resulting wood is utilized, resulting in the designations white-, brown- and soft-rot fungi. Soft-rot fungi include the Ascomycetes and Fungi Imperfecti, which decompose cellulose while lignin is partially degraded. Brown-rot fungi include some of the Basidiomycetes, and have a preference for hemicellulose and cellulose but will degrade lignin by demethylation. Lignin remains undegraded, so an amorphous, brown, crumbly residue is the product. The third group is the white rot fungi (WRF) that include some of the Basidiomycetes. Lignin is degraded more extensively and rapidly by WRF than by any other known group of organisms. In contrast to most other fungi and bacteria, WRF are capable of completely degrading lignin to carbon dioxide and water (Scklarz et al., 1989).

Lignin-degrading fungi are a group of taxonomically heterogeneous higher fungi, which are widely distributed in a range of environments from tropical to temperate. Microorganisms in this group are mainly saprophytes that belong to the division Eumycota, subdivision Basidiomycotina (Burdsall, 1998). They are characterized by their ability to depolymerize and mineralize lignin efficiently using extracellular ligninolytic enzymes. Many classes of extracellular enzymes have been implicated in lignin degradation: lignin peroxidases (LiPs), manganese peroxidases (MnPs), H_2O_2 producing enzymes and laccases. A review of various WRF has demonstrated that the peroxidases and laccases are secreted by almost all ligninolytic fungi, however the enzymes may be differentially expressed in different species and even between various strains

(de Jong et al.; 1992; Esposito et al., 1991; Hatakka, 1994; Nerud et al., 1991; Orth et al., 1993; Palaez et al., 1995; Szklaez et al., 1989, Waldner et al., 1988). One of the most intriguing questions is which enzyme or enzyme combination is responsible for the most efficient lignin depolymerization and degradation.

Two of the best examined lignin-degrading fungi are *P. chrysosporium* and *T. versicolor*. *P. chrysosporium* is a ligninolytic basidiomycete which has been found to be a good degrader of lignin. It has been used as a model organism to study physiological requirements for lignin biodegradation. It was found that only during secondary metabolism, triggered by nitrogen, carbon or sulphur limitation, do liquid cultures of *P. chrysosporium* produce ligninolytic enzymes (Erwin et al., 1993). It was found to produce high levels of ligninolytic peroxidases, including lignin and manganese peroxidase, in response to nutrient depletion. For a long time, it was believed that *P. chrysosporium* did not produce laccase, because activity is repressed by glucose. However, laccase activity has been detected constitutively at a basal level in *P. chrysosporium* (Perez et al., 1996; Srinivasan et al., 1995). Most of the knowledge of the ligninolytic peroxidases has been determined from studies of *P. chrysosporium*.

T. versicolor is another well studied WRF, that secretes substantial amounts of laccase. It is also known as *Coriolus versicolor* and *Polyporus versicolor*. Similarly to *P. chrysosporium*, *T. versicolor* secretes both LiP and MnP. Unlike *P. chrysosporium*, *T. versicolor* secretes substantial levels of laccase. Low concentrations of several laccases are produced constitutively during growth on wood, while higher concentrations are induced by the addition of aromatic compounds such as xylidine and ferulic acid. *T. versicolor* is the most studied laccase-producing fungus and most of the knowledge of laccase has been determined from these studies (Call and Mucke, 1997).

C. gallica is a white rot fungus that secretes both MnP and laccase but not LiP. It produces high volumetric activity levels when grown by solid state fermentation on oat bran. Its laccase activity has been correlated with decolorization of some industrial dyes (Rodriguez et al.,1999).

2.2 Wood Composition.

Wood and plant material are composed mainly of three structural polymers which are cellulose, hemicellulose and lignin. Cellulose determines the basic morphology of wood cell walls beingapproximately 45% of the weight of wood. Glucose units in cellulose are linked by β-1,4-glycosidic bonds in a linear polymer. Microfibrils are bundles of highly ordered cellulose molecules that are surrounded by lignin and hemicellulose, which gives wood its mechanical strength and protection from microorganisms (Kirk and Cullen, 1998).

Hemicellulose is 25% to 30% of the weight of wood. Similar to cellulose, the backbone of hemicellulose is linear β-1,4-linked monosaccharides, however the hemicellulose is shorter than cellulose polymers. Hemicellulose has three basic forms: 1,4 -β-D-xylans, 1,3- and 1,4-β-D-galactans, and 1,4-β-D-mannans (Sarikaya et al., 1997). Side groups such as sugars and acetyl esters are present which impart a gel-like characteristic to hemicellulose (Kirk and Cullen, 1998).

Lignin makes up 30% of the weight of wood. A typical lignin contains 10%-20% phenolic groups (Youn et al. 1995). Lignin is a rigid organic polymer which has evolved for preservation purposes, giving plants structural integrity and providing protection from microorganisms (Call and Mucke, 1997). Lignin physically protects cellulose and hemicellulose from enzymatic hydrolysis. Due to the heterogeneous nature of lignin, it is resistant to degradation.

The structure of lignin (Figure 1) is different from that of cellulose and hemicellulose being composed of branched substituted phenylpropane units joined by a random distribution of stable carbon-carbon and ether linkages (Youn et al. 1995, Kirk and Cullen, 1998). The polymer consists of aromatic compounds of three *p*-hydroxycinnamyl alcohols including *p*-coumaryl (7, below), coniferyl (1, below) and sinapyl alcohol (13, below) (Youn et al. 1995). Lignin is highly branched and heterogeneous, being made up of phenyl-

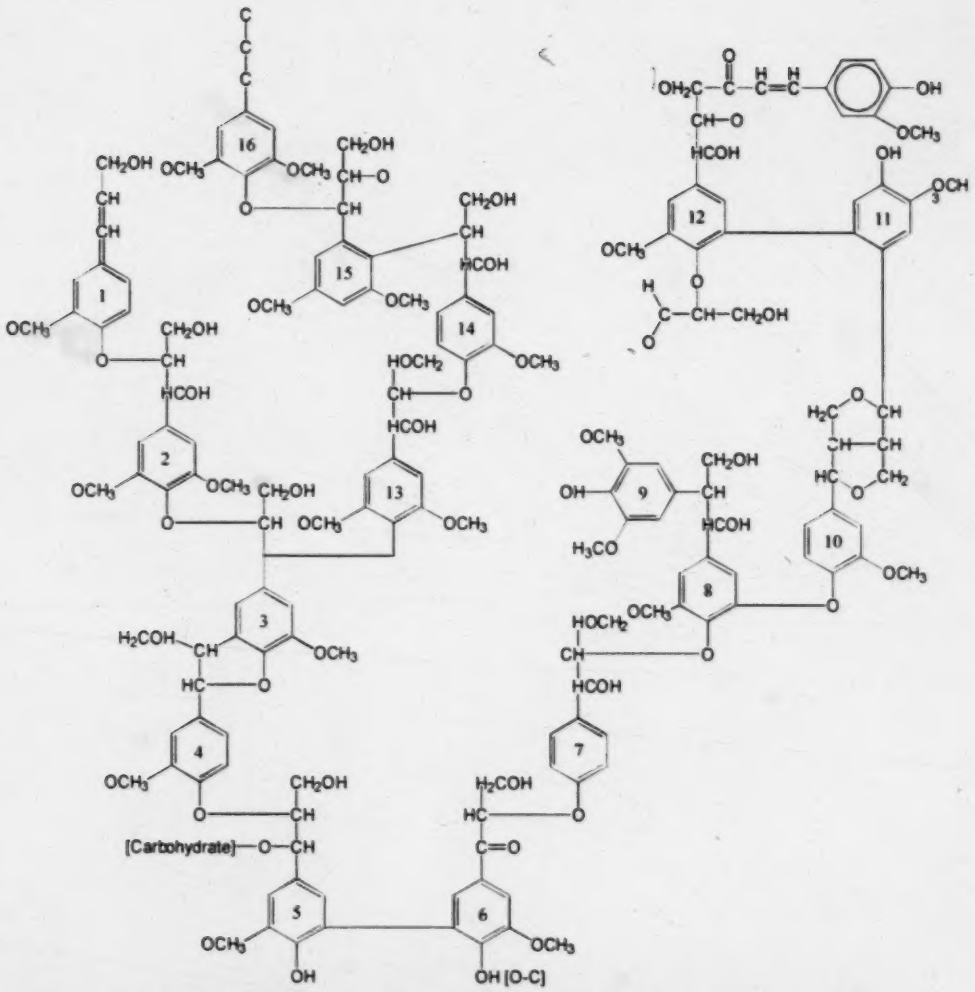


Figure 1. A hypothetical structure for lignin (after Call and Mucke, 1987).

propanoid units which are linked through a variety of different bonds of which the most abundant linkage is guaiacylglycerol-β-arylether (β-O-4), shown linking units 1 and 2 (Youn et al. 1995). Other linkages include diarylpropane (β-2;) between units 14 and 15, pinoresinol (β-β') between units 10 and 11, and diphenyl ether (4-O-5') between units 8 and 10 (Youn et al. 1995).

2.3 Ligninolytic enzymes.

Lignin molecules are too large to be transported into the cell. As well, because of the heterogeneous structure of lignin, it is essential that extracellular enzymes are involved in lignin biodegradation. The degradation mechanism is oxidative, as interunit carbon-carbon and ether bonds are present in the structure. Lignin is stereoirregular, so the mechanism must be less specific than typical degradative enzymes. These requirements are fulfilled by the extracellular peroxidases and oxidases of WRF that act nonspecifically by generating unstable free radicals that are able to undergo a variety of spontaneous cleavage reactions (Kirk and Cullen, 1998).

Enzymes that play a role in ligninolysis are LiP, MnP and laccase. These catalyze single electron oxidations to produce a free radical that undergoes a variety of nonenzymatic coupling substitutions and fission reactions explaining the chemical changes in lignin during degradation by fungal cultures. These enzymes have been shown to utilize low molecular weight mediating substrates to perform lignin degradation. Not all WRF produce all of these enzymes, each fungus produces its own unique combination of enzymes and has a characteristic ability to degrade lignin (Kirk and Cullen, 1998).

2.3.1 Lignin Peroxidase.

Lignin peroxidase has been detected in *P. chrysosporium* cultures and a number of other WRF such as *T. versicolor, Bjerkandera adusta, Panus tigrinus, Pleurotus ostreatus* and *Phlebia radiata* (Kirk and Farrel, 1987), *Phanerochaete*

magnoliae, Lentinula edodes, Phellinus pine, Trametes hirsutus and Trametes gibbossa (Hatakka, 1994).

Typically, LiPs are glycosylated enzymes of approximately 40 kDa with acidic isoelectric points (pIs) and pH optima and a single ferric protoporphyrin IX heme group (Kirk and Cullen, 1998). Lignin peroxidase is a typical peroxidase in that it undergoes a cyclic reaction mechanism, as seen in Figure 2. H_2O_2 oxidizes LiP to a two electron deficient intermediate termed Compound I. To return to a resting state, two 1 electron oxidations of donor substrates are performed. This results in an unstable cation radical, which can react to form a variety of degradation products (Kirk and Cullen, 1998). These enzymes are powerful oxidants, more so than typical peroxidases. For this reason LiPs can oxidize the usual peroxidase substrates, such as phenols and aromatic amines. Due to this powerful oxidizing ability, aromatic ethers and PAHs with appropriate ionization potentials that are not typical peroxidase substrates can also be oxidized by LiP (Kirk and Cullen, 1998). Lignin peroxidase oxidizes both phenolic and nonphenolic components of lignin and is therefore thought to be an effective enzyme in the degradation of lignin.

2.3.2 Manganese Peroxidase.

Several fungi have been found to secrete MnP including *P. chrysosporium* (Leisola et al., 1987), *P. tigrinus* (Maltseva et al., 1991), *P. radiata* (Karhunen et al., 1990), *L. edodes* (Forrester et al., 1990), *Phanerochaete sordida* (Ruttiman-Johnson et al., 1994), and *T. versicolor* (Johansson and Nymar, 1993), among others. This wide distribution among ligninolytic fungi suggests that MnP may also play a key role in the process of lignin degradation. These enzymes are glycosylated, are 36-60 kDa and have acidic pIs and pH optima (Kirk and Cullen; 1998; Y. Wang, unpublished). Similar to LiPs, MnPs have a conventional catalytic cycle. However, MnP uses H_2O_2 to oxidize Mn^{2+} to Mn^{3+}, as seen in Figure 3. Simple organic acids stimulate MnP activity because they stabilize the Mn^{3+} and promote its release

$$LiP + H_2O_2 \rightarrow LiPI + H_2O$$

$$LiPI + Ar \rightarrow LiP + LiPII + Ar^{+\cdot}$$

$$LiPII + Ar \rightarrow LiP + Ar^{+\cdot} + H_2O$$

Figure 2. The catalytic cycle of LiP. Ar = aromatic reducing substrate, LiPI = one electron oxidized enzyme, LiPII = two electron oxidized enzyme (Gold and Alic, 1993).

$$MnP + H_2O_2 \rightarrow MnPI + H_2O$$

$$MnPI + Mn(II) \rightarrow MnPII + Mn(III)$$

$$MnPII + Mn(II) \rightarrow MnP + Mn(III) + H_2O$$

Figure 3. The catalytic cycle of MnP. Ar = aromatic reducing substrate, MnPI = one electron oxidized enzyme, MnPII = two electron oxidized enzyme (After Gold and Alic, 1993).

from the enzyme. White rot fungi produce suitable metal chelating agents such as oxalic or malonic acids to chelate Mn^{3+}. The resulting Mn^{3+}-chelates diffuse extracellularly to mediate oxidation of lignin at a distance from the enzyme. This enzyme specifically oxidizes phenolic compounds in the presence of H_2O_2. Mn^{3+} oxidizes phenolic rings to phenoxy radicals, leading finally to the decomposition of aromatic compounds (Kirk and Cullen, 1998). However the oxidizing ability of Mn^{3+} chelates is not sufficient to oxidize nonphenolic substructures of lignin therefore extensive degradation of lignin is not likely (Kirk and Cullen, 1998).

Other studies have shown that MnP can produce diffusible oxyradicals by another mechanism. The peroxidation of unsaturated lipids is promoted by MnP with Mn^{2+}. This produces transient lipoxyradicals which have sufficient oxidizing power to oxidize nonphenolic compounds and have been shown to oxidize nonphenolic lignin model compounds. This system has been shown to depolymerize both phenolic and phenol-blocked (methylated) synthetic lignins, indicating that the system could function to depolymerize lignin (Bogan and Lamar, 1995). The role of MnP in lignin degradation is quite well understood.

2.3.3 Peroxide-generating enzymes.

Both peroxidases require an extracellular source of H_2O_2 for their reactions to occur. There are several extracellular enzymes that could fill this role. Glyoxal oxidase (GLOX) is an extracellular enzyme that can oxidize small aldehydes such as glyoxal and methylglyoxal, both of which are metabolites produced by *P. chrysosporium*. The end result of the reaction is to transfer electrons to O_2 generating H_2O_2. However, GLOX is not produced by all WRF therefore other enzymes must also play a role in H_2O_2 production (Kirk and Cullen, 1998).

Aryl alcohol oxidase (AAO) is another extracellular enzyme that is a candidate for H_2O_2 generation. AAO is an FAD-dependent oxidase that has been detected and isolated from different ligninolytic fungi. The oxidation of aryl

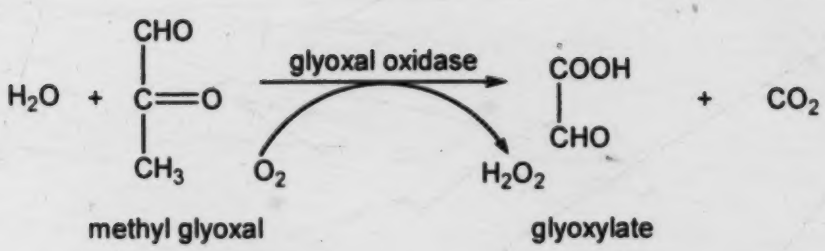


Figure 4. Production of H_2O_2 for lignin degradation.

α- and α-β-unsaturated alcohols to aldehydes occurs concomitantly with the reduction of O_2 to H_2O_2 as seen in Figure 4 (Marzullo et al., 1995). AAO transfers electrons to O_2 to produce H_2O_2 by oxidizing benzyl alcohols to aldehydes. Benzyl alcohols are secreted by WRF such as *B. adusta* (Kirk and Cullen, 1998). An example of an AAO is veratryl alcohol oxidase that is secreted by *P. ostreatus*, which performs the reduction of veratryl alcohol to veratrylaldehyde (Marzullo et al., 1995; Ander and Marzullo, 1997).

2.3.4 Laccase.

Another major group of enzymes involved in lignin degradation is the laccases. Laccases are widely distributed in the plant kingdom and are particularly abundant in many lignin-degrading WRF (Mayer, 1987). Yoshida discovered laccases when he observed that latex of *Rhus vernicifera* (Japanese or Chinese lacquer tree) rapidly hardened upon exposure to air. Upon purification the enzyme was named laccase (Call and Mucke, 1997). As the years have progressed, laccases have been detected in more than 60 fungal strains and higher plants but only in a few bacteria such as *Azospirillum lipoferum* (Alexander et al., 1996; Gianfreda et al., 1999).

Laccase is one of a small group of enzymes called the large blue copper proteins or blue copper oxidases. Large numbers of oxygenases and oxidases are known to use molecular oxygen as one of their substrates but of these six can reduce oxygen to two molecules of water. Cytochrome C oxidase, a heme/Cu containing enzyme and the blue oxidases, such as L-ascorbate oxidase, ceruloplasmin, bilirubin oxidase and phenoxazinone synthase are able to perform this reaction along with laccase (Call and Mucke, 1997). Each has a Cu atom as an important prosthetic group. However, all these enzymes differ markedly in their primary amino acid sequence and their biological function. They are rather similar in their three dimensional structure and their characteristic Cu binding in three different types of sites (Messerschmidt and Huber, 1990; Messerschmidt et al., 1992).

2.4 Laccase function.

2.4.1 Laccase in morphogenesis.

Laccases have been widely studied over the years in terms of their function in nature. Laccases have been proposed to have distinct roles in some fungi that do not appear to be related to ligninolysis. Fungi utilize laccases in synthetic reactions such as oxidative polymerization (Call and Mucke, 1997). *Aspergillus nidulans* produces two laccases involved in oxidative polymerization for pigment formation. The green color of the conidia and the pigment in the hull cells are a result of such reactions (Clutterbuck, 1972).

Rhizomorphs are mycelial strands formed from large numbers of tightly adpressed hyphae. These structures are formed in conjunction with laccase synthesis in *Armillaria mellea*. The laccase may be synthesized to produce a polyphenolic glue which could act to glue hyphae together. (Thurston, 1994)

Fruiting body development may also be dependent on laccase activity in some fungi. *Schizophyllum commune* strains secrete high levels of laccase at the time they develop fruiting bodies (De Vries et al., 1986). *Agaricus bisporus*, a cultivated button mushroom, also has laccase activity. Laccase activity accumulates during vegetative growth along with mycelial mass but is rapidly inactivated at the beginning of fruiting body formation. (Thurston, 1994).

2.4.2 Laccase in plant pathogenic processes.

Laccases are widely distributed in plants and have been implicated in the phytopathogenicity of several fungi. Endogenous phenols are oxidized to quinones that are toxic to microorganisms, which provide a role for laccases in a defense mechanism against pathogens. They are also associated with the browning of food and used for the removal of natural phenols (Gianfreda et al., 1999; Call and Mucke, 1997).

Many horticultural plants can be infected with *Botrytis cinerea*, a laccase producer, which causes soft rot in plants such as carrots and cucumbers. Similarly, it can cause 'noble rot' and 'grey rot' of grapes. Cucurbitacins and

tetracyclic triterpinoids produced by cucumbers protect the plant from infection, as they will specifically repress laccase synthesis by the fungus. This suggests that this fungal laccase belongs to the enzyme system involved in the infection process (Bar-Nun and Mayer, 1989, 1990). If these crops have a defense mechanism against laccase it seems plausible that the laccase could be involve in the pathogenic process (Thurston, 1994).

2.4.3 Other roles of laccase.

Laccase, LiP and MnP are all able to polymerize aromatic compounds *in vitro*. In this respect it is plausible that this is a defense mechanism. Compounds that may be toxic, are low molecular weight and are phenolic are polymerized, resulting in sufficiently sized products that are not able to enter cells. Thus the potential threat is neutralized. Laccase also efficiently scavenges of radicals, thus neutralizing any threat posed by the radical (Call and Mucke, 1997).

Laccase may play an important role in soil humus synthesis. Microorganisms, mainly WRF, will cleave plant biopolymers, including lignin, into their monomeric structural units. Laccases can then transform polyphenols to quinones. Numerous different types of compounds, such as amino compounds, will react with quinones to produce increasingly complex humic substances (Gianfreda et al., 1999; Stevenson, 1994).

2.4.4 Lignification.

Laccases in woody tissues have been proposed to be part of the lignin synthesizing system due to their polymerization ability. On the other hand, laccases in WRF have been proposed to be part of the lignin degrading system (Gianfreda, 1999).

Lignin peroxidase and manganese peroxidase are able to cleave key bonds in a range of compounds that resemble the immense diversity of compounds that make up lignin, thus it seems that these enzymes will degrade

lignin (Thurston, 1994). The same argument cannot be used to determine the definitive role of laccase in ligninolysis. Ander and Eriksson (1976) showed that *Sporotrichum pulverulentum* (*P. chrysosporium*) mutants, that are laccase deficient, have a decreased ability to degrade lignin. Revertants with laccase activity showed the ability to degrade lignin. Additional evidence is that laccase has the ability to take part in some of the reactions of ligninolysis. The abundance of laccase-producing WRF seems to indicate that the main role of fungal laccases is to depolymerize lignin (Thurston, 1994).

There are some data that indicate that laccase activity does not correlate with ligninolysis. *Fomes annosus* laccase can be inhibited by thioglycollic acid but this compound does not affect the growth of the fungus on lignin or its ability to cleave high molecular mass lignosulphonate (Thurston, 1994). When the laccase of *T. versicolor* was inhibited by thioglycollic acid lignin degradation continued (Thurston, 1994).

From the evidence put forward it is clear that a part of the mechanism of laccase in lignin degradation is unknown. It has been shown that laccase can cleave a significant proportion of the structures found in lignin, however the definitive role of laccase in ligninolysis is unresolved. The classical action of laccase oxidation results in the polymerization of phenols or the formation of quinones. The fact that laccase tends to polymerize phenolic substrates *in vitro* does not eliminate the possibility that it contributes to lignin breakdown, as MnP and LiP also tend to polymerize substrates *in vitro* (Thurston, 1994). Polymerizing activity can be prevented when mediating substrates, such as 2,2'-azino-bis-(3-ethylbenzthiazoline sulfonate) (ABTS), are present (Bourbonnais and Paice, 1990, 1992).

Further evidence has been put forward for the definitive role of laccase in lignin degradation. The redox potentials of known laccases are too low to oxidize the nonphenolic components of lignin directly. *Pycnoporus cinnabarinus* produces a compound, 3-hydroxyanthranilate, that can mediate the oxidation of nonphenolic compounds of lignin by overcoming this barrier (Eggert et al.,

1996). This is how laccase could possibly function for complete depolymerization of lignin. Another study demonstrated that a number of compounds either produced by the fungus or present during degradation of lignocellulose substrates, were able to mediate the oxidation of nonphenolic aromatics by *T. versicolor* laccase, including 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (Johannes and Majcherczyk, 2000). It has been proposed that, in the presence of appropriate substrates, WRF are capable of oxidizing both phenolic and nonphenolic substructures of lignin and therefore could play a role in delignification that is equally as significant as that of LiP.

2.5 Properties of laccase enzymes.

2.5.1 Physical properties of laccases.

Large numbers of laccases have been studied and several general properties have emerged along with their variations and some singular properties. The selection of information presented here is not intended to be comprehensive. Laccases are blue Cu-containing oxidases. All laccases detected to date are glycoproteins. Glycosylation of laccase is believed to play a role in secretion, susceptibility to proteolytic degradation, Cu retention and thermal stability (Li et al., 1999). Upon purification, laccase enzymes demonstrate considerable heterogeneity. Glycosylation content and composition of fungal glycoproteins can vary with growth medium composition (Hashimoto and Pickard, 1988). For this reason data can be heterogeneous.

Laccase are typically 60-80 kDa, of which about 15%-20% is carbohydrate. The sugar composition has been analyzed in only a few examples, such as *Podospora anserina* and *B. cinerea* (Call and Mucke, 1997) . Other general properties of laccases and properties for a specific laccase from *Trametes* are presented in Table 1.

2.5.2 Copper content and properties.

Laccases contain three types of Cu that can be distinguished by their

Table 1. Properties of laccases.

PROPERTY	RANGE FOR LACCASES	LACCASES OF TRAMETES
pH optimum	3.0-7.5	3.6, 3.8, 4.0, 5.0, 5.3
Temperature optimum (°C)	40-80	60
Molecular mass (kDa)	60-390	60-65
Number of amino acids	NA	520-550 (including N-terminal secretion peptide about 20 aa)
Carbohydrate content(%)	10-45	14 (4 N-linked carbohydrate chains)
Prosthetic groups: Cu content (atoms per molecule)	2-16	4
Isoelectric point	2.6-7.6	~3-3.5
Number of isoenzymes	up to 5	2-3

After Call and Mucke, 1997

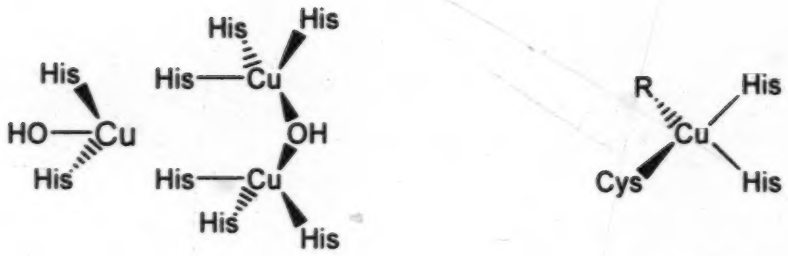


Figure 5. Active site arrangement of Cu atoms in laccase

spectroscopic and paramagnetic properties. Type 1 Cu atoms confer the intense blue colour on these enzymes (Thurston, 1994). The 'blue Cu site' exhibits extensive cysteine sulfur to Cu^{2+} charge transfer transition at 610 nm (Call and Mucke, 1997). Laccase also contains type 2 and type 3 Cu atoms. The crystallization and x-ray structure of *Coprinus cinereus* laccase has been determined (Ducros et al., 1998). Various physical characteristics plus the crystal structures of *Zucchini* ascorbate oxidase and human serum ceruloplasmin were used to postulate the positions of the Cu atoms in laccase. The type 2 and type 3 Cu atoms in laccase form a trinuclear cluster (Ducros et al., 1998). The crystal structure shows the type 2 Cu atoms are disposed almost equidistant from the type 3 Cu atoms. The binuclear pair is 3.4 Å apart and 3.9 Å and 4.0 Å from the type 2 Cu atom (Messerschmidt et al., 1989; Messerschmidt and Huber et al., 1990). A schematic diagram of the Cu content of the laccase is shown in Figure 5. The catalytic center of fungal laccase is becoming well understood.

Purified laccases have variations in the measured number of Cu atoms, ranging from two to four Cu atoms per enzyme subunit (Thurston, 1994). Lower values should be considered with caution. Laccase may be damaged by degradative enzymes and these damaged laccases may not retain all of their Cu atoms (Thurston, 1994). It has been demonstrated experimentally that Cu atoms in proteins are firmly bound but the type 2 Cu atom can be selectively removed (Reinhammer and Malmstrom, 1981). Such a depletion may also occur during purification. It was also demonstrated that type 1 Cu centers lack a methionine in fungal laccases. Methionine can act as a Cu binding ligand which provides a stabilizing factor for the Cu centre in laccase. All characterized fungal laccases have been shown to lack the methionine (Karlsson et al., 1989). For this reason it is thought that laccases have relatively unstable Cu centers. This could also account for Cu content measurements that are lower than expected.

The primary amino acid sequences of ascorbate oxidase and laccase show only low similarity. *C. hirsutus*, *P.radiata*, and *Agaricus bispora* laccase

gene sequences have been published (Rojima et al., 1990; Saloheimo et al., 1991; Perry et al., 1993). They all encode polypeptides of ~520-550 amino acid residues that include N-terminal secretion peptides. There is commonality in the one cysteine and ten histidines that bind to the Cu atoms. There is also a small amount of sequence around the four binding areas that is similar. The Cu binding amino acids and their place in the sequence are conserved in the gene sequences. These properties closely resemble those of ascorbate oxidase. However *N. crassa* laccase and the *P. radiata* laccase amino acid sequences indicate that laccase can fold into a three domain β-barrel structure similar to that of ascorbate oxidase (Thurston, 1994). An ascorbate oxidase model has been used for the prediction of the three-dimensional structure of laccase. The amino acids proposed for the coordination of the three types of Cu ions are highly conserved in most laccases and other blue Cu oxidases. One cysteine, ten histidine residues and probably one leucine are involved in the binding of Cu in each laccase molecule. The absence of methionine in type 1 Cu binding sites of all laccases may contribute to the more positive redox potential of the Cu center of laccases compared to other type 1 Cu centers (Thurston, 1994).

2.5.3 Catalysis by laccase.

To function, laccase depends on Cu atoms distributed among the three different binding sites. Cu atoms play an essential role in the catalytic mechanism. There are three major steps in laccase catalysis. The type 1 Cu is reduced by a reducing substrate, which itself is oxidized. The electron is then transferred internally from type 1 Cu to a trinuclear cluster made up of the type 2 and type 3 Cu atoms. The O_2 molecule is reduced to water at the trinuclear cluster. Kinetic, spectroscopic and EPR data show that types 1 and 2 Cu atoms are involved in electron capture and transfer and that the type 2 and type 3 Cu atoms are involved in oxygen binding (Gianfreda, 1999).

The O_2 molecule binds to the trinuclear cluster for asymmetric activation and it is postulated that the O_2 binding pocket appears to restrict the access of

oxidizing agents other than O_2. H_2O_2 is not detected outside of laccase during steady state laccase catalysis indicating that a four electron reduction of O_2 to water is occurring (Gianfreda, 1999). A one-electron substrate oxidation is coupled to a four-electron reduction of oxygen so the reaction mechanism cannot be straightforward. Laccase must operate as a battery, storing electrons from individual substrate oxidation reaction to reduce molecular oxygen (Call and Mucke, 1997). In fact, it appears that bound oxygen intermediates are also involved (Gianfreda, 1999). Details of the O_2 reduction have not been fully elucidated and continue to be studied.

In a typical oxidation of a substrate by laccase (Figure 6), there is a one-electron reaction that generates a free radical which is typically unstable. The free radical has several options for further reaction. A second enzyme-catalyzed oxidation may take place which converts a phenol to a quinone. Non-enzymatic reactions such as polymerization can occur to produce amorphous insoluble melanin-like products (Thurston, 1994). Laccases use oxygen as the electron acceptor to remove protons from the phenolic hydroxyl groups. This reaction gives rise to phenoxy radicals that can spontaneously rearrange, which can lead to fission of carbon-carbon or carbon-oxygen bonds of the alkyl side chains, or to cleavage of aromatic rings (Marzulla et al., 1995; Salas et al., 1995).

The redox potentials of fungal laccases range from 0.4 to 0.8 V (Gianfreda, 1999). The redox potential of the type 3 Cu pair in *T. versicolor* laccase is around 0.8 V. Five enzymes and forty substrates were used in a study to show that oxidation can be controlled by the redox potential differences between the reducing substrates and the type 1 Cu in laccase (Xu, 1996). It was shown that the higher redox potential of laccase correlates with higher activity. As well, it was concluded that structural differences in the substrate active site (blue Cu) control the redox potential and thus the substrate specificity (Xu et al., 1996). To increase oxidation, either the redox potential of substrate should be lower or the redox potential of laccase at the type 1 Cu site should be higher

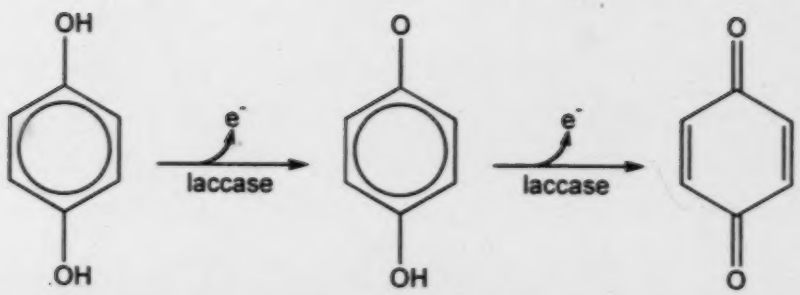


Figure 6. A typical laccase reaction. A diphenol undergoes a one electron oxidation to form an oxygen centred free radical. This can be converted to a quinone in a second enzyme catalyzed reaction.

(Gianfreda et al., 1999). It is clear from these studies that catalysis by laccase is not straightforward and there is still much to be discovered at the molecular level.

2.5.4 Substrate specificity of laccase.

Enzymes in general are highly specific but all lignolytic enzymes are nonspecific, including laccase. Laccase is an important oxidant for aromatic rings substituted with electron donating groups, such as phenolics and aromatic amines. These are the preferred electron rich substrates of laccase. There is some difficulty in defining laccase by its reducing substrate. Laccase has an overlapping substrate range with tyrosinase, another type of Cu-containing oxidase, but laccase does not oxidize tyrosine itself (Thurston, 1994). Thus laccases are non-specific regarding to their reducing substrate, and the range of substrates oxidized varies from one laccase to another (Thurston, 1994).

Structure and redox potential of a compound define the substrate of ligninolytic enzymes, including laccase. Lignin peroxidases are able to oxidize substrates of extremely high electropotential, up to $E_{1/2}$=1.49 V whereas MnPs are able to oxidize substrates up to $E_{1/2}$ = 1.12 V. Laccase cannot oxidize non-phenolics with a higher electropotential than $E_{1/2}$= 1.06 V (Call and Mucke, 1997).

The reducing substrate spectrum for laccase is diverse as long as the redox potentials are not too high (>1V) (Gianfreda et al., 1999). While laccase has low specificity for its reducing substrates, it has a strong preference for its oxidizing substrate, O_2 (Gianfreda et al., 1999). Thus laccase can oxidize *o*- and *p*-diphenols, aminophenols, methoxyphenols, polyphenols, polyamines, lignin, some organic ions, aryl diamines and a considerable range of other compounds (Thurston, 1994; Call and Mucke, 1997; Gianfreda et al., 1999). Presently, the full range of laccase substrates is not known and still less is known about the range of compounds that laccase activity can affect directly or indirectly.

The pH optimum of laccase depends on the substrate being studied.

Oxidation of substrates that do not involve proton exchange will be affected by the pH in that laccase activity will decrease with an increase in pH. Oxidation that involves proton exchange will be affected by a change in pH differently. Laccase can display an optimal pH that depends on the enzyme rather than the substrate (Bourbonnais and Paice, 1992; Fukushima and Kirk, 1995; Xu, 1996). This is the case for the oxidation of phenols which have an optimal pH range of 3 to 7 for fungal laccases (Xu, 1996). This pH activity profile occurs because of the redox potential difference between a reducing substrate and the type 1 Cu. This correlates with the electron transfer rate and is favored for a phenolic substrate by higher pH. However a hydroxide anion can bind to the type2/type3 Cu which inhibits the activity at higher pH (Gianfreda et al., 1999).

2.5.5 Mediating substrates.

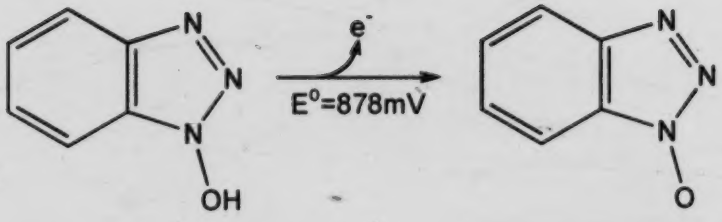
Laccase is produced by many WRF that do not produce LiP. As LiP has been implicated in the degradation of nonphenolic components, these fungi must have another mechanism to degrade the nonphenolic components of lignin because the redox potentials of known laccases are too low to oxidize directly the non-phenolic components of lignin. It has been shown that the artificial laccase substrate, ABTS, has the capacity to act as a mediating substrate enabling the oxidation of non-phenolic compounds that are not laccase substrates on their own because they have higher redox potentials than laccase (Bourbonnais et al., 1998). One of the first reports of a mediated system was by Bourbonnais and Paice (1990) using the laccase from *T. versicolor*. It was shown that laccase was able to cleave a nonphenolic dimer in the presence of ABTS. The presence of the mediating substrate, ABTS, prevented and reversed the polymerization of kraft lignin by *T. versicolor* laccase (Bourbonnais et al., 1995). *Pycnoporus cinnabarinus* is a laccase-producing fungus that degrades very efficiently both the phenolic and nonphenolic components of lignin. The redox potential barrier for nonphenolic compounds can be overcome by 3-hydroxyanthranilate, a metabolite produced by *P. cinnabarinus*, which has been

shown to mediate the oxidation of non-phenolic substrates by laccase (Eggert et al., 1996). However, in the presence of ABTS, one of the best substrates for laccase, the non-phenolic, nonlaccase substrate veratryl alcohol was converted to veratrylaldehyde (Call and Mucke, 1997). Thus there is ample evidence for the role of mediating substrates in the activity of laccase.

All laccase mediating substrates discovered to now are substrates of laccase (Call and Mucke, 1997). More than 100 possible mediating substrates have been described but the most common are HBT and ABTS (Johannes and Majcherczyk, 2000). Synthetic mediating substrates are heterocyclic compounds belonging to the general classes of phenoxazinones, phenothiazines or phenoxybenzothiazoles (Eggert et al., 1996). Activity of laccase-mediating substrate systems towards compounds depends on a combination of two main factors: the redox potential of the enzyme and the stability and reactivity of the radical generated by oxidation of the mediating substrate (Bourbonnais et al., 1998).

Several hypotheses have been proposed for the mechanism of mediating substrate systems. The first hypothesis is that the mediating substrate can act as a redox mediating substrate, i.e. reversible. It is thought that laccase oxidizes the mediating substrate and this oxidized form of the mediating substrate can oxidize the substrate, and is consequently reduced back to its non-oxidized form: the species responsible for the oxidation of the substrate would be the oxidized mediating substrate (Li et al., 1998). Another hypothesis is that active intermediates are generated during the oxidation of the mediating substrate by laccase. These intermediates can abstract hydrogen. In such a reaction the mediating substrate would be continuously consumed (Li et al., 1998) and not be reversible. According to the results of the study performed by Li et al. (1998) the first hypothesis is more probable.

Further studies into the mechanism of mediating substrates have elucidated the chemical species and proposed detailed mechanisms. The potential and producing species that are potent electrophiles (Bourbonnais et



HBT nitroxyradical of HBT

Figure 7. Formation of the nitroxy radical of HBT by laccase.

mechanism of laccase mediation has been investigated for HBT. There was one species of HBT with a redox potential of 878 mV for HBT/HBT^{+} as seen in Figure 7. The nitroxy radical intermediate was not stable and decayed rapidly but was shown to catalyze the oxidation of veratryl alcohol to veratraldehyde. The nitroxy radical formed when HBT is oxidized by laccase is a potent electrophile that easily abstracts hydrogen. So it is clear that mediating substrates work to increase the substrate range of laccase by increasing the difference in redox al., 1998).

The mechanism of laccase mediation has also been investigated for ABTS. The oxidation of ABTS by laccase produces the stable dark green cation radical, ABTS^{+} and then the dication (ABTS^{2+}) is formed. Both oxidized species were relatively stable and the reactions are highly reversible. The redox potential for ABTS/ABTS^{n+} was 472 mV and 885 mV for ABTS^{+}/ABTS^{2+}. It was observed that the dication was the intermediate responsible for the oxidation of non-phenolic compounds such as veratryl alcohol. The cation radical was shown to react only with the phenolic structures. *T. versicolor* laccase has a redox potential of 585 mV. This is 300 mV below the redox potential of ABTS^{+}/ABTS^{2+} couple. Laccase can slowly oxidize ABTS to ABTS^{2+} provided that the reaction is driven forward by the subsequent reaction of the dication with veratryl alcohol or some other compound (Bourbonnais et al., 1998). It is clear that the enzyme must produce ABTS^{2+} for the oxidation of nonphenolic compounds to occur but oxidation by ABTS^{+} is limited to phenolic compounds. ABTS oxidation is the basis of the common laccase assay, used in these studies.

An effective mediating substrate system should have many features. An effective laccase must have a high Kcat for an effective laccase mediating substrate with high redox potential and must be resistant to inactivation by the free radical form of the laccase mediating substrate. As well, the free radical must be able to effectively oxidize nonphenolic compounds. There are several

methods to make a laccase/mediating substrate system more effective such as modification of the laccase structure to increase its Kcat and to improve its stability to the free radical attack. A more effective laccase mediating substrate would be another way to enhance lignin degradation (Li et al., 1998).

2.5.6 Inhibition of laccase.

Laccase can be strongly inhibited by a variety of compounds. Halides, such as fluoride, bromide and chloride , as well as azide, cyanide and other small anions are inhibitory to laccase. These will bind to the type 2 and type 3 Cu cluster to interrupt the internal electron transfer. Inhibition by specific anions is a function of the laccase. Each laccase can be inhibited by different compounds to a different extent. This may be explained by the accessibility of anions to the opening of the channel of the trinuclear Cu cluster (Xu, 1996). Many other compounds are able to inhibit laccase. At alkaline pH laccase is inhibited by the hydroxyl ion. Metal ions such as Hg^{2+},as well as fatty acids, sulfhydryl reagents,and quaternary ammonium detergents whose reactions may involve amino acid residue modification, Cu chelation, or conformational change can all inhibit laccase (Gianfreda et al., 1999).

2.6 Production of laccase.

For biotechnological and environmental purposes and for a better understanding of the properties of laccase, crude and purified laccases must be available in large amounts. Screenings of WRF have been performed to identify the most efficient laccase-producing strains. As well, many studies have been performed to select the most suitable culture medium to optimize enzyme production by a particular strain. This requires the development of reproducible inexpensive medium that enhances enzyme production (Collins et al., 1997; Gianfreda et al., 1999, Kaal et al., 1993; Mester et al., 1996; Pickard et al., 1999).

2.6.1 Production by addition of chemical compounds.

Factors affecting laccase production by a particular strain include the nature of inducers or the time of induction. Other considerations are the composition of culture medium and the growth conditions.

When an inducer is included several factors are important. The chemical nature, the amount, and the time of addition of the inducer can influence laccase production. Many compounds have been tested and shown to improve laccase production. Phenolic compounds that are related to the natural substrate lignin or lignin derivatives are good inducers, however, nonligninolytic compounds were also discovered to be effective enhancers of laccase production (Gianfreda et al., 1999). Depending on the fungus, some may act to increase laccase production, may have no effect or may serve to decrease laccase production. One of the most common laccase inducers is 2,5-xylidine. This has variable effects on laccase production by different fungi: it stimulated enzyme formation by organisms such as *P. ostreatus* and *T. versicolor*, however enzyme activity of *B. cinerea* remained unchanged and laccase production by *Podospora anserina* was markedly inhibited (Gianfreda et al., 1999).

Other aromatic compounds have been tested for their ability to stimulate laccase production in fungi, with varying response. When added to *P. radiata* cultures, benzyl alcohol caused production of relatively high amounts of laccase (Niku-Paavola et al., 1990; Rojalski et al., 1991). Other compounds tested as inducers include substituted phenols, such as *p*-methoxyphenol and 4-aminophenol, acids such as ferulic acid and syringic acid and anilines such as *o*- and *p*-anisidine. *o*-Anisidine caused a 62-fold increase in laccase production by *T. versicolor* (Fahraeus and Tullander, 1956). Thus there are many chemical compounds that can be tested to enhance laccase production.

2.6.2 Production by addition of lignin sources.

Natural and synthetic lignins have been shown to enhance laccase production by many fungi. Culture conditions and medium composition can also

play a major role in the level of laccase expression. Grown in the presence of kraft lignin, *P. cinnabarinus* and *Cyathus bulleri* displayed an increase in laccase activity 3 and 1.2 times higher than without lignin, respectively (Gomez-Alarcon et al., 1989; Vasdez and Kulad, 1994). Extracts from cotton stalk, barley straw, cotton-wheat straw mixtures, potato wastes, malt extract, and corn straw were tested as natural lignins to stimulate laccase production and there was increased laccase formation in several fungi, including *P. ostreatus* and *T. versicolor* (Gianfreda et al., 1999). Veratryl alcohol, a lignin related compound, is a substrate of laccase and it stimulated the production of laccase in *P. radiata* , *P. cinnabarinus* and *T. versicolor* by two to three times more than cultures lacking veratryl alcohol. *T. versicolor* grown on both wheat straw and beechwood showed stimulated laccase activity as high as 3.5 fold in comparison with growth on glucose (Schlosser et al., 1997). *Ceriporiopsis subvermispora* when grown on a wheat bran medium showed 10-fold higher laccase activity over production in salts medium (Salas et al., 1995). Increased laccase levels are produced by several of these fungi when grown in solid state fermentation on whole oats including *T. versicolor* and *P. chrysosporium* (Rodriguez et al., 1999). There is ample evidence that natural lignin sources are able to induce laccase activity. There are many natural lignin stimulators for laccase production to choose from to try to enhance laccase production in new fungal strains.

2.7 Large-scale production of laccase.

The laboratory to large-scale transition requires an economical method for production of the product. One way to perform this is to scale-up from shake flasks to larger stirred tank reactors. An advantage of fermentors is the ability to closely control environmental factors such as pH and dissolved oxygen. These are difficult to control in shake flasks but can be optimized for growth of fungus in the reactors. A disadvantage of fermentors can be viscosity, which can depend on the physical characteristics of the fungal growth, either mycelial or pelleted, and the sensitivity of the cells to shear forces. Oxygen may not be

supplied sufficiently for microbial growth in a viscous culture. If the culture is shear sensitive, agitation must be limited leading to a limited oxygen supply. One way to reduce viscosity is to grow the fungus in a pelleted form (Jones, 1998). There are many advantages for large-scale production; nonetheless, with every process there are disadvantages.

There are many different fermentor designs available but the stirred tank reactor is the most common type used for large scale production because it has the most flexibility for production of most cell types including bacteria, fungi and algae. Physical properties of fungi can be handled in this type of reactor by several methods. Changing the impeller type or adding pure oxygen to meet dissolved oxygen requirements can be beneficial in some cases. Small-scale reactors have been scaled-up and reproducibly operated at volumes of up to 200 000 L for the past 40 years (Jones, 1998).

The ultimate aim of large-scale production is to reproducibly carry out the fermentation. Several aspects of fermentation must be maintained for the success of the process. The master cell bank must be preserved. This is a standard reference culture and is a reliable source for working cell banks (WCBs) which represent the first step in large scale production. Many WCBs are prepared to reduce disaster from contamination. Each of the WCBs is tested for quality control in terms of purity. WCBs are a short-term storage system (Jones, 1998).

Before a fermentation system can be utilized, preliminary trials should be performed in shake flasks because this can provide beneficial information about the growth characteristics of the organism being studied. A vast number of parameters can be tested in a shorter period of time and a minimum of expense is incurred. The main advantage of such studies is that experiences with shake flasks allows the researcher to become accustomed to the organism, and this basic knowledge can be used to full advantage during the fermentation studies (Jones, 1998).

The inoculum train is an important consideration when performing large-

scale fermentations. An inoculum train is used to build up sufficient biomass at the correct phase of growth so that, upon inoculation of the reactor, rapid growth of the organism will occur in the production medium. The parameters surrounding this process must be intensely studied to optimize growth of the microorganism in the fermentor (Jones, 1998).

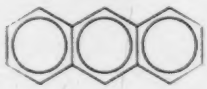
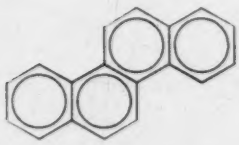
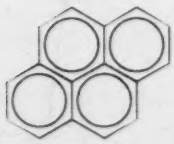
Raw materials will influence the cost-effectiveness of a large-scale fermentation. Crude raw materials are relatively inexpensive sources which are rich in vitamins, trace metals, and other growth factors that must be added separately in synthetic growth medium. Common sources of sucrose are sugar beet and sugarcane. Suitable nitrogen sources are yeast extract, corn steep liquor, or cottonseed extracts (Jones, 1998). All of these sources are effective in supporting microbial growth. It is clear that producing fungus on a large scale can be done effectively using fermentors.

2.8 Polycyclic aromatic hydrocarbons.

2.8.1 Structure and properties.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that occur in soils, sediments, airborne particles, fresh water and marine environments (Bumpus, 1989). PAHs are nonpolar, neutral, organic molecules that comprise two or more fused benzene rings arranged in various configurations, including linear, angular and clustered alignments (Collins et al., 1996). Some examples of PAHs are shown in Table 2 along with some of their properties. This table indicates that these compounds range from two to five fused benzene rings. The carcinogenicity of PAHs tends to be a function of their size. As the PAH increases in molecular weight, its carcinogenicity also increases. As well, as the molecular weight increase there is a concomitant decrease in water solubility which indicates the potential for accumulation (Bezalel et al., 1996). PAHs bind to soils and sediments and persist in the environment (Bezalel et al., 1996). The distribution and fate of these pollutants in the environment are of increasing interest because PAHs have been found to

Table 2. PAHs and some of their properties.

PAH	Structure	Water solubility (mmol/L)	Ionization Potential (eV)
Acenaphthene		2.9×10^{-2}	7.70
Anthracene		3.7×10^{-4}	7.45
Phenanthrene		7.2×10^{-3}	8.03
Chrysene		1.3×10^{-5}	7.80
Pyrene		7.2×10^{-4}	7.72

have toxic, mutagenic and carcinogenic properties and for this reason are a major concern in the environment. Their presence poses a serious threat to animals, including humans.

2.8.2 Sources and exposure.

PAHs originate from a variety of sources. Natural and anthropogenic pyrolysis of organic matter such as fossil fuels are sources (Moen and Hammel, 1994). Vehicle emissions, coal tar residues, creosote for wood preserving, incomplete combustion of fossil fuels and forest fires are all important contributors of PAHs to the environment (Kotterman et al., 1996; Field et al., 1996). Industrial effluents from coal gasification and liquefaction processes, waste incineration and petroleum also contribute to PAH contamination in terrestrial, aquatic and marine sediments (Cerniglia and Yang, 1984). Due to their poor aqueous solubility, PAHs released into the environment are found deposited in and bound to soil and eventually enter the ground water from there. From the sources above, one can see that PAHs have contributed to widespread contamination of the environment.

Humans can be exposed to PAHs in several ways. Inhalation of tobacco smoke and polluted air, ingestion of contaminated foods, exposure to environmental contamination in soil and water are all means of exposure. Workers in several industries may be exposed through contact with creosote, oils, tars and asphalt (Sutherland et al., 1995).

2.8.3 Removal of PAHs.

2.8.3.1 Physical removal.

Due to the toxicity of PAHs and their widespread distribution in the environment they constitute a health threat. For this reason techniques are needed to remove PAHs from contaminated environmental sites such as soils and waters. A variety of processes can be implemented to eliminate PAHs from the environment including volatilization, adsorption, chemical oxidation,

photodecomposition and biodegradation (Cerniglia, 1993).

Physical processes eliminate some of the contaminants present, but other more effective techniques need to be employed to remove residual PAHs. Due to the high cost of trapping or removing PAHs from the environment, the potential of using microorganisms for decontamination and detoxification of PAH polluted sites is under investigation (Sack et al., 1997).

2.8.3.2 Biological removal.

There have been many attempts to use bioremediation of PAHs to eliminate PAHs from the environment. Eukaryotic microorganisms, such as fungi, cannot use PAHs as a sole carbon source for growth but usually cometabolize the PAH to dead end metabolites. In contrast, bacteria can completely degrade many PAHs and use them as the sole carbon and energy source for growth (Sutherland et al., 1995).

At present, many microorganisms are known to metabolize the lower molecular weight PAHs but these PAHs tend not to be highly carcinogenic. Less is known about the potential for biodegradation of higher molecular weight PAHs, which tend to be more carcinogenic (Cerniglia, 1993). A microorganism's ability to degrade PAHs is dependant on the bioavailability of the compound. Naphthalene, biphenyl, anthracene, and phenanthrene all have been shown to be utilized as a sole carbon source.

Laboratory demonstration of the degradation of many PAHs by microorganisms has been reported, yet these compounds persist in the environment. Poor bioavailability limits degradation and this is caused primarily by their low water solubility, a low dissolution rate and strong adsorption to the soil matrix (Cerniglia, 1993). Other factors influencing bioremoval of PAHs from soils are environmental and microbial factors. These include soil type, moisture content, oxygen content, concentration of the PAH, redox conditions, sediment toxicity, temperature, pH, electron acceptors, organic matter content, seasonal factors, the presence of PAH-degrading microorganisms, inorganic nutrient

availability, depth, diffusion and physicochemical properties of the PAH (Cerniglia, 1993). Optimization of these factors can increase degradation but the main factor is bioavailability of the PAH for microbial metabolism: degradation may remain slow unless bioavailability is enhanced.

2.8.3.2.1 Bacteria.

Upon review of the literature, three general patterns for the degradation of PAHs have been identified. These include the metabolism of PAHs to *cis*-dihydrodiols, *trans*- dihydrodiols or quinones as seen in Figure 8. The crucial step in their biodegradation is oxidative fission of the fused aromatic ring. In the first mechanism, mainly used by bacteria, dioxygenase catalyses the incorporation of both atoms of O_2 into the aromatic nucleus. This reaction is the major mechanism for oxidation that leads to the formation of *cis*-dihydrodiols. The resulting dihydrodiols are transformed to diphenols which can be further modified by cleavage with other dioxygenases to achieve ring fission (Sutherland et al., 1995; Cerniglia, 1993). Further processes produce tricarboxylic acid cycle intermediates (Cerniglia, 1993). Most information about PAH degradation has been obtained from the study of bacteria.

2.8.3.2.2 Nonligninolytic fungi.

A diverse group of ligninolytic and nonligninolytic fungi also have the ability to oxidize PAHs. Many of the end-products produced by fungal cometabolism are not as toxic as the parent compound. For this reason this mechanism is considered detoxification. PAHs are not their sole carbon and energy sources (Sutherland, 1992). Nonligninolytic fungi and some bacteria such as *Streptomyces* and *Mycobacterium* (Tongpim and Pickard, 1999) use cytochrome P-450 monooxygenases to catalyze the incorporation of one atom of molecular oxygen into the aromatic nucleus and reduce the remaining atom to H_2O. While the resulting arene oxide intermediate is carcinogenic, it can undergo further metabolism by epoxide hydrolase to form a *trans*-dihydrodiol or

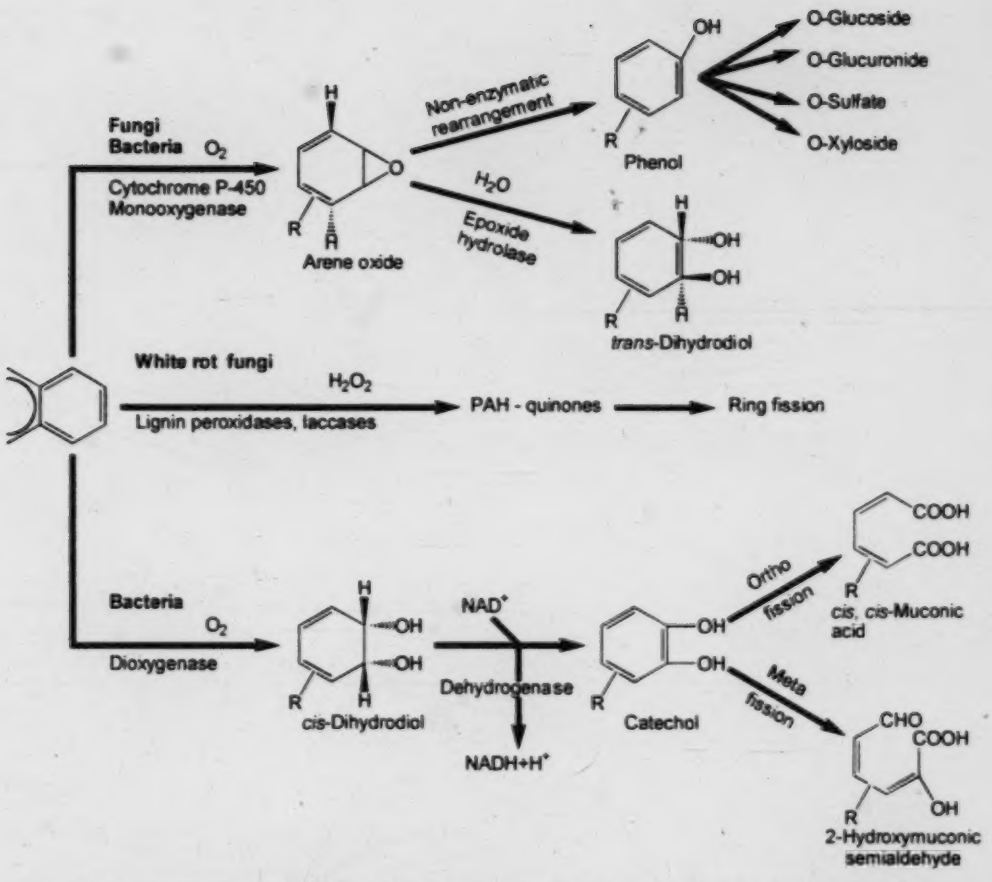


Figure 8. Pathways of microbial degradation of PAHs (After Cerniglia, 1993).

undergo non-enzymatic rearrangement to produce a phenol which can form many conjugates as seen in Figure 8. The toxicity of these conjugates, is unknown but is thought to be low (Sutherland,1992; Cerniglia, 1993). Microorganisms that have only these pathways cannot utilize PAHs as carbon sources but do detoxify PAHs. However, these *trans*-dihydrodiols are more soluble and fungi may therefore potentiate further availability of PAHs to bacteria.

2.8.3.2.3 White rot fungi.

Highly condensed PAHs are degraded to a limited extent intracellularly. This is due to low solubility and restricted transport of these PAHs through the cell membrane (Eggen and Majcherczyk, 1998). One way to overcome this limitation is through extracellular mechanisms, such as those possessed by WRF. Nonspecific extracellular enzymes are efficiently produced by WRF and can oxidize PAHs during growth on carbohydrates (Sutherland, 1992). Some WRF metabolize PAHs to their quinones and other metabolites that do not appear to involve a *cis* or *trans* dihydrodiol (Sutherland, 1992). Lignin peroxidases may initially produce a quinone from which several pathways may follow resulting in ring fission (Sutherland et al., 1995). Metabolites with a higher water solubility and chemical reactivity than the parent PAH are produced by WRF. It was thought that mono- or dihydroxylated PAH metabolites could be substrates for bacteria and that degradation reactions in soil caused by indigenous microflora will proceed (Cerniglia, 1993). It has been shown that both fungi and bacteria are able to break down PAH diones (Andersson and Henrysson, 1996). Other enzymes secreted by WRF that may be involved in PAH degradation are MnP and laccase. The roles of these enzymes have been examined at some length in the literature. Numerous experiments have shown that this group of organisms is able to degrade mixtures of even highly condensed PAHs, including benzo[a]pyrene. Other xenobiotics that have been successfully degraded by WRF include environmental contaminants such as

DDT, nitroaromatics, chlorophenols and polychlorinated biphenyls (Bogan et al., 1996; Eggen and Majcherczyk, 1998).

Among the WRF displaying ligninolytic activity, *P. chrysosporium*, *B. adusta*, *T. versicolor* and *P. ostreatus* have been closely studied in terms of PAH degradation (Bezalel et al., 1996). Degradation has been studied in experiments using liquid cultures but the applicability of this system has also been demonstrated in contaminated soils (Eggen and Majcherczyk, 1998). The degradation of a wide array of PAHs by *P. chrysosporium* including phenanthrene, fluorene, benzofluorene, anthracene, fluoranthene, pyrene, benz[a]anthracene and benzo[a]pyrene has been reported (Bogan and Lamar, 1995). However, the rates of PAH oxidation are rarely correlated with any ligninolytic enzyme activities (Collins et al., 1996). In contrast to nonligninolytic fungi, WRF are also able to mineralize PAHs as the ligninolytic mechanism is involved in later steps of metabolism leading to CO_2 evolution.

2.8.3.2.3.1 Role of lignin peroxidase and manganese peroxidase.

There are several possible mechanisms involved in PAH degradation by WRF. As mentioned, the oxidative enzymes of WRF include LiP, MnP and laccase. Both peroxidases are able to oxidize substrates that have high redox potential because these enzymes have active sites that are more electron deficient than other peroxidases (Vazquez-Duhalt et al., 1993). Lignin peroxidase can directly catalyze a one electron oxidation of PAHs that have an IP (IP) value of ≤7.55 eV, which produce quinones that can be further metabolized by ring fission (Hammel et al., 1992). The first enzyme suspected in PAH degradation was LiP. For example, the first step in anthracene oxidation by *P. chrysosporium* is catalyzed by LiP (Hammel et al., 1991). Thereafter it was observed that some PAHs that were not LiP substrates were degraded by WRF. This is exemplified by the degradation of phenanthrene. It was cleaved to 2,2-diphenic acid (Hammel et al., 1991). This led researchers to the possible role of MnP.

Manganese peroxidase is thought to work through a similar mechanism of a one electron oxidation with the diffusible oxidizing agent Mn^{3+}-chelate, which is a weak oxidant (Bohmer et al., 1998). However it was incapable of oxidizing PAHs with ionization potentials equal to or greater than chrysene (≤ 7.8 eV). Thus it was not clear that Mn^{3+} mediated oxidation could explain the degradation of PAHs such as phenanthrene and fluorene. Then an *in vitro* system mediated by the peroxidation of unsaturated lipids by MnP and Mn^{3+} was observed. This system was shown to catalyze the oxidative cleavage of phenanthrene to diphenic acid, therefore lipid peroxidation-based cooxidation was used to explain phenanthrene degradation by fungal cultures (Moen and Hammel, 1994). A similar mechanism of lipid peroxidation was involved in fluorene oxidation *in vivo* (Bogan et al., 1996).

2.8.3.2.3.2 Role of laccase and mediating substrates.

The role of laccase in PAH degradation has been less well studied. Laccase can also catalyze a one electron oxidation of PAHs such as anthracene and benzo[a]pyrene that both have ionization potentials ≤ 7.55 eV. Although the lignin degrading ability of the WRF *P. ostreatus* correlates closely with its laccase activity, no link between laccase activity and the PAH oxidizing ability of this fungus could be established (Bezalel et al., 1996; Collins et al., 1996). It was later shown that laccase has a role in PAH oxidation by WRF. It was demonstrated that crude enzyme preparations as well as two purified isoenzymes from *T. versicolor* were able to oxidize anthracene and benzo[a]pyrene. Direct oxidation of anthracene by the two purified laccases was observed but a marked increase in levels of oxidation occurred when ABTS was present. In contrast, no significant direct oxidation of benzo[a]pyrene by purified laccase was observed. The presence of ABTS in the reaction mixture was essential for high levels of benzo[a]pyrene oxidation (Collins et al. 1996). It must be remembered that anthracene and benzo[a]pyrene have low IPs and that PAHs with higher IPs have not been shown to be oxidized.

The activity of laccase is restricted to compounds with low ionization potentials such as aromatic compounds with a phenolic functional group (Bohmer et al., 1998). The substrate range of laccase extends to nonphenolic lignin structures when mediating substrate compounds such as ABTS are present. The mediating substrate presumably functions as a diffusible redox mediating substrate between that compound and the enzyme. It has been demonstrated that such laccase/mediating substrate couples oxidize PAHs and that the IP threshold value for the oxidation of PAHs by laccase appears to be similar to that of LiP (Bohmer et al., 1998). The oxidation of PAHs with IP beyond the oxidative ability of LiP has not been reported but phenanthrene was efficiently oxidized by laccase in the presence of either HBT or unsaturated lipids (Bohmer et al., 1998; Pickard et al., 1999).

There is no direct contact between the substrate and enzyme when the oxidation of PAHs is mediated by the action of the mediating substrate compounds in their oxidized state. The single step process proceeds by the abstraction of one electron or hydrogen atom. These reactions involve a large difference between the oxidation potentials of the radicals or cations of the mediating substrates. For example $ABTS^{2+}$ has a redox potential of 1.09V, and the PAH has a potential of up to 1.55 V. As a result these reactions are thermodynamically unfavorable. However such reactions can be possible if a follow up process irreversibly removes one of the products from the equilibrium. This also applies to the oxidation of ABTS and HBT by laccases. Redox potential is not the only factor that can affect oxidation of PAHs. Steric hindrance, their electronic structures, or the formation of complexes with the oxidant can all have an impact on the oxidation of PAHs (Johannes and Majcherczyk, 2000).

Most effective mediating substrate compounds are products of chemical syntheses that are expensive or toxic. Natural mediating substrates would be an ideal solution but these have still not been shown to play a role in natural systems. As some WRF secrete only laccases and these are important in wood

degradation, there is an indication that mediating substrates are present in natural systems and therefore have a natural origin. In a study by Collins et al. (1996), ultrafiltrate from fungal broth was added to the reaction with laccase of *T. versicolor* and anthracene. There was a significant increase in the level of oxidation thus indicating that a potential natural mediating substrate was present. This can be further supported by the lack of correlation between laccase activity and the biodegradation of PAHs by laccase producing fungi. It can be theorized that natural mediating substrates could be typical laccase substrates. This is supported by the role of ABTS and HBT which are both laccase substrates. It is thought that the natural mediating substrates are produced by fungi (Johannes and Majcherczyk, 2000).

Making the assumption that all radicals formed by laccase oxidation can potentially act as a mediating substrate to act on nonsubstrate compounds such as PAHs, Johannes and Majcherczyk (2000) designed a study to look at phenolic mediating substrates. Phenol, hydroxyquinone, and aniline are some of the simplest laccase substrates and were found to be mediating substrates. The oxidative ability of phenols increased with a less negative difference between the oxidation potentials of phenoxy radicals and the desired PAH. One of the best phenolic type mediating substrates tested was 4-hydroxybenzoic acid. This compound, together with numerous aromatic compounds, is produced and secreted extracellularly by WRF. Some of the compounds such as 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde were also found to be active as mediating substrates in the oxidation of PAHs. 4-Hydroxybenzoic acid very effectively mediated the oxidation of PAHs such as benzo[a]pyrene and displayed an oxidation pattern comparable to that of the artificial synthetic mediating substrate, HBT (Johannes and Majcherczyk, 2000). A physiological role for mediating substrates in the oxidation of the PAHs had not been proved *in vivo* but with this evidence it is plausible that natural mediating substrates may play a role in the degradation of PAHs by laccase-producing WRF. The presence of a laccase utilizing natural mediating substrates could explain the

ability of laccase producing fungi to metabolize PAH extracellularly and the lack of a direct correlation between the enzyme activity and degradation (Johannes and Majcherczyk, 2000).

2.9 Enhancement of enzyme activity.

2.9.1 Why use organic solvents?

Enzymatic catalysis in organic solvents has opened a new field of biotechnological applications of enzymes. The ability to use enzymes in nonaqueous solvents greatly expands the potential scope and economic impact of biocatalysis. When biological catalysts are placed in this unnatural environment they exhibit a number of remarkable novel properties such as altered stereo-selectivity, enhanced stability and increased rigidity (DeSantis and Jones, 1999).

There are many reasons to justify the use of organic media instead of aqueous solutions when working with enzymes. In the presence of an organic solvent, poorly water soluble compounds can be used in higher concentrations and this could shift the reaction equilibrium (Vazquez-Duhalt et al., 1993). The solubility of PAHs has always been a limiting factor in their degradation as mass transfer limitations occur in aqueous systems. The addition of solvents to the mixture will aid in solubilization of the PAHs. There could be a shift in reaction equilibrium when water is one of the products because of a substantial reduction in water (Brink et al., 1988). There is also facilitated recovery of products and biocatalyst (Torres et al., 1997). As well in the presence of organic solvents there is less risk of microbial contamination (Brink et al., 1988).

Not all of the advantages could be relevant for one particular bioconversion and there are also some important side effects. The biocatalyst may be denatured or it may be inhibited by the organic solvent. There is also the increasing complexity of the reaction system. The underlying problem associated with most systems is cost and the organic solvent will introduce additional costs (Brink et al., 1988). It appears that the advantages are much

more appealing and would outweigh any potential disadvantages for use of enzymes in the presence of organic solvents.

2.9.2 Enzyme catalysis in organic solvents.

The catalytic properties of enzymes are exhibited to the full extent when enzymes are in their native conformation. This conformation in solution is determined by a network of hydrogen bonds, van der Waals forces, electrostatic interactions and hydrophobic interactions (Khmelnitsky et al., 1988). Interactions will arrange correctly when the enzyme molecule has a definite hydration shell. Water molecules are attached to the protein surface mainly by hydrogen bonds. Organic solvents can cause loss of the hydration shell, or a strong distortion of the shell by disrupting the hydrogen bonds. This can upset the system of interactions supporting the native conformation which can lead to loss of conformation resulting in a loss of catalytic properties (Khmelnitsky et al., 1988). The hydration shell must be preserved to retain enzyme catalytic activity in media with organic solvents. As the amount of water-miscible organic solvent increases in aqueous solutions there is a concomitant decrease in enzyme activity. This has been shown to correlate with changes in protein structure (Torres et al., 1996).

Upon exposure to a non-aqueous medium, enzymes can be affected by many factors that are able to destroy the enzymes' aqueous structure and function. Physical characteristics of organic solvents have been used to try to predict the effect of organic solvents on enzyme catalysis. Such a parameter is the logarithm of the partition coefficient of the enzyme between octanol and water (log P) (Halling, 1990; Lanne et al., 1987, Manjon et al., 1992). However this applies only to pure organic solvents containing low amounts of water. The physical properties of organic solvents cannot be applied to aqueous mixtures containing water miscible solvents and, therefore other parameters must be considered. A hydrophobicity parameter for water-organic solvent mixtures has been proposed. This is called the hydrophobicity concept (H) and was

conceptualized using the substrate partitioning between the active site of the enzyme and the bulk solvent. An increase in this parameter would mean an increase in solvent hydrophobicity, which could lead to substrate partitioning away from the active site. This would cause less substrate to interact with the active site, which could cause a decrease in activity of the enzyme (Torres et al., 1997). However this partitioning effect would also depend on the hydrophobicity of the enzyme and its active site.

2.9.3 Chemical modification of enzymes.

One of the challenges for the practical implementation of protein biocatalysts is to increase stability and activity, particularly in nonaqueous or hydrophobic environments. Activity of some enzymes, in the presence of organic solvents, can be enhanced by increasing the hydrophobicity of the enzyme (Vazquez-Duhalt et al., 1995). Chemical modifications of the enzyme surface have been performed to improve the catalytic activity of lignin peroxidase in organic solvents (Tinoco and Vazquez-Duhalt, 1998). The modifications are employed to change the superficial characteristics of the enzyme molecules.

There are various kinds of modifiers, which interact with specific amino acids in proteins. Chemical modification has two major objectives. One objective is to determine structures of proteins such as active sites of enzymes and the states of amino acids (Inada et al., 1986). As well these modifications can alter or improve the native function of proteins and endow them with useful new functions (Inada et al., 1986). In addition to amphipathic groups such as polyethylene glycol, aromatic moieties have been covalently bonded to the enzyme surfaces to alter their hydrophobicity and catalytic properties (Vazquez-Duhalt et al., 1995). Covalent chemical modification, one of the original methods available for altering protein properties has reemerged as a powerful tool for tailoring proteins and enzymes.

2.9.3.1 Protein cross-linking.

Over the years several methods have been employed to change some property of an enzyme in hopes of finding a novel catalytic function. Protein crosslinking was one of the first methods used to modify proteins. Crosslinked enzyme crystal (CLEC) formations are highly active, easy to handle and are stable at elevated temperatures and in organic solvents (Govardhan, 1999). Inter- and intra-molecular crosslinking of enzymes with bi- and poly-functional reagents have stabilizing effects on proteins. This process involves crosslinking enzyme microcrystals with bifunctional monomers, such as glutaraldehyde which is an efficient crosslinker and is most often used (Govardhan, 1999). This method has been used to produce more stable enzymes. The use of glutaraldehyde for enzyme crosslinking was employed to stabilize carboxypeptidase for x-ray structure determinations. It was observed that the crosslinked enzyme retained ~ 5% residual activity. Crosslinked thermolysin had enhanced stability that also retained high levels of enzymatic activity (DeSantis and Jones, 1999). CLECs have been shown to be two to three orders of magnitude more stable than their soluble counterparts (Govardhan, 1999). The stability is derived from protein-protein interactions that occur in the crystal and are maintained by the CLECs. The crosslinking lends additional stability by preventing unfolding of the enzyme (Govardhan, 1999). Protein crosslinking is a promising tool for modifying proteins to increase stability and retain enzymatic activity in harsh environments such as in the presence of organic solvents.

2.9.3.2 Monofunctional polymers.

Another method for modification is chemical modification with monofunctional reagents. This permits the binding of specific monomeric or polymeric functionalities. In particular, covalent modification with the amphiphilic polymer polyethylene glycol (PEG) has been employed.

PEG has both hydrophilic and hydrophobic properties. The hydrophilic nature makes it possible to modify enzymes in an aqueous solution and the

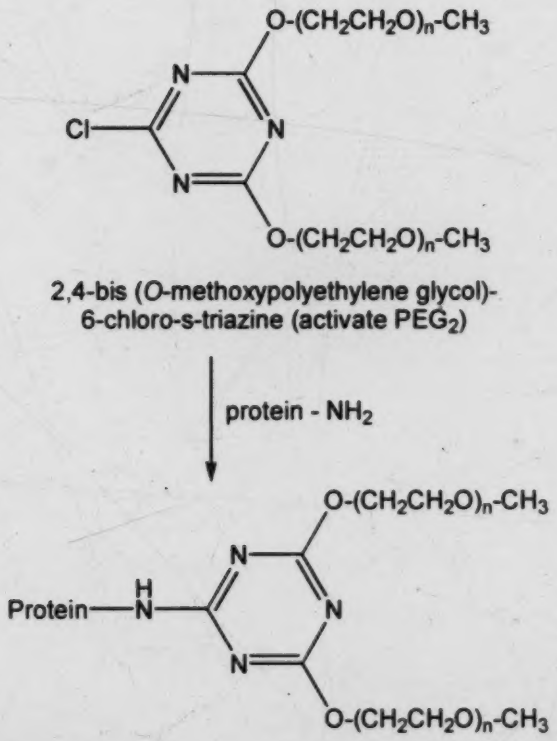


Figure 9. Chemical reaction for modification of proteins with polyethylene glycol moieties.

hydrophobic nature enables modified enzymes to function in a hydrophobic environment (Inada et al., 1986). Activated PEG can be used to modify free amino groups located on the surface of the protein under mild conditions, at room temperature in an aqueous environment. This is to avoid denaturation of the enzyme. Free amino groups can be found at the N-terminal amino acid and on amino acids such as lysine or arginine. The chemical reaction that may be used is diagrammed in Figure 9 (Inada et al., 1986). In the reaction 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine (PEG-CN) has one chlorine atom that can react with free amino groups. Two chains become attached to the surface of the protein through the triazine ring. The molar ratio of PEG-CN can be altered to control the degree of modification (Inada et al., 1986). This modification has been used to solubilize proteins for applications in organic solvents such as benzene or toluene (DeSantis and Jones, 1999). Modified enzymes exhibit high enzymatic catalysis in organic solvents. PEG- modified catalase and peroxidase efficiently catalyse their respective reactions in organic solvents (Inada et al., 1986). PEG-modification of cytochrome c has increased the catalytic activity in systems containing organic solvents. Cytochrome c was PEGylated and subsequently subjected to esterification of its carboxylate groups to produce an improved biocatalyst. Although the native protein could, in the presence of hydrogen peroxide, oxidize eight of the twenty PAHs evaluated, the modified cytochrome c oxidized 17 of the 20, under the same conditions (Tinoco and Vazquez-Duhalt, 1998).

PEG-modified enzymes in organic medium can retain activity because PEG chains are strongly hydrated. These can create an aqueous shell around the enzyme which can protect it from denaturation by organic solvent. In fact, PEG-modified peroxidase and catalase in benzene solution have the same absorption spectrum as the native enzymes in aqueous solutions (Khmelnitsky et al., 1988). It seems that PEG holds promise to improve catalytic abilities of enzymes in organic solvents.

2.9.3.3 Addition of a small moiety.

Another method is to chemically introduce small molecular functionalities onto the protein. Ribonuclease A was chemically coupled to D-glucosamine producing mono- and di-glycosylated enzyme which increased specific activity by 80% compared to the unmodified enzyme (DeSantis and Jones, 1999).

Methylation is another possible modification for enhanced functionality. Methylation is not site specific: all free carboxylic groups could be modified with this technique. The reaction should be performed in organic solvents without the presence of water. Methylation of cytochrome c showed the same substrate pattern as the unmodified cytochrome c (Tinoco and Vazquez-Duhalt, 1998).

It is possible that the different methods of chemical modification of proteins can be combined to further enhance enzyme functionality. Many trials can lead to the best combination of modification for better stability and functionality. It is also possible to vary the amount of modification with carefully controlled chemical reactions. It is clear that there is a place for protein engineering using chemical modification in the future of protein biochemistry.

3. Materials and Methods.

3.1 Culture Methods.

3.1.1 Microbiological.

Coriolopsis gallica UAMH 8260 was obtained from the University of Alberta Microfungus Mold Herbarium, Devonian Botanical Gardens, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9. The fungus was grown on potato dextrose agar (Difco, Detroit, Michigan) at 28°C for 5 days before being stored at 4°C. Maintenance cultures were transferred every 3 months.

3.1.2 Growth medium and incubation conditions.

C. gallica inocula were prepared in glucose-malt extract-yeast extract medium (GMY) modified as described by Mester et al. (1996). This medium contained (per liter): 10 g of glucose, 3.5 g of malt extract (Difco), 2.5 g of yeast extract (Difco), 2.0 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot H_2O$. Inocula were prepared by aseptically homogenizing 1 cm^2 portions of the surface mycelium from potato dextrose agar plates in 50 ml portions of GMY with an Omnimixer (Sorvall, Norwalk, Connecticut.) for 10 s at top speed. After 3 days growth in 500 ml shake flasks at 200 rpm and 28 °C, the cultures were homogenized, and a 5% to 10% inoculum (depending on density of growth, approximately 2 mg dry weight) was used to inoculate production medium. Variations in this basic growth protocol are mentioned in the text where applicable.

3.2 Analytical techniques.

3.2.1 Spectrophotometric assays.

3.2.1.1 Laccase assay.

Laccase activity was determined by the oxidation of ABTS (Wolfenden and Wilson, 1982), in a reaction mixture containing 1 mM ABTS in 0.1 M sodium acetate buffer, pH 4.5, with a 5 to 50 µl enzyme or culture supernatant sample. The oxidation was followed at 30°C and at 436 nm, ε_{436} = 29300 M^{-1} cm^{-1} for ABTS. Activity was expressed in units defined as 1 µmol of substrate oxidized

per minute at 30°C. Single activity values were taken during growth and purification but triplicate values were used during characterization. Replicates did not deviate more than 10%.

3.2.1.2 Reducing sugar content assay.

To each diluted 1 ml sample, containing up to 375 μg of sugar, 1 ml of mM dinitrosalicylic acid was added and mixed well. The tubes were heated at 90°C for 10 min, cooled to room temperature and 5 ml of dH_2O were added and mixed. The absorbance was read at 550 nm and glucose was used as the standard (Ghose, 1987).

3.2.1.3 Protein determination assay.

Protein determination was done by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The range utilized was 12 to 100 μg.

Standard or sample (800 μl) solution was pipetted into a test tube. Dye reagent concentrate (200 μl) was added to each tube and mixed well. These were incubated at room temperature for at least 5 min but not longer than 60 min. The absorbance was measured at 595 nm (Bradford, 1976).

3.2.1.4 Free amino group determination.

Enzyme (150 μl, 90-150 μg protein) was added to 150 μl of a 4% sodium carbonate solution (pH 8.5) and 150 μl of a 0.1% solution of 2,4,6-trinitrobenzene sulfonic acid. This was reacted for 2 h at 40°C. After the incubation, 150 μl of 10% sodium dodecyl sulfate and 75 μl of 1.0 N HCl were added. The absorbance was determined at 335 nm (Habeeb, 1965).

3.3 Production medium development.

Various preparations of possible lignin sources were tested for their ability to enhance laccase production. Each source was prepared as 2% (w/v) in 100

ml of 60 mM phosphate buffer and incubated at 28°C, at 200 rpm on a rotary shaker, after inoculation. GMY medium was included as a control. Each source was tested in triplicate. Samples (1 ml) were removed, clarified in a microfuge at 13000 rpm for 2 min and laccase activity was determined. Variations on this experiment included comparing bran flakes from various commercial sources; testing the particle free supernatant as a source of soluble lignin inducers; adjusting the bran flakes content (1%-4% w/v); altering the medium pH (pH 4-6.5) and changing aeration level.

3.4 PAH mineralization.

Mineralization experiments were carried out using [U-^{14}C]naphthalene, [9-^{14}C]phenanthrene, [9-^{14}C]anthracene and purified [9-^{14}C]phenanthrene. Approximately 150,000 dpm of each PAH or 50,000 dpm of purified phenanthrene were added to each sterile 100 ml sidearm flask to a final concentration of 1 mM or 0.3 mM. Cultures grown in production medium for 5 days were introduced into the flasks and mediating substrates, ABTS and HBT were introduced to a final concentration of 1 mM. Sterile controls for each PAH were included to detect abiotic degradation and volatilization of the PAH. GMY grown cultures were included as a control. The evolution of $^{14}CO_2$ from radiolabelled PAH was measured by the method of Fedorak et al. (1982). Samples of liquid (1 ml) and headspace gas (2 ml) were withdrawn by syringe and replaced by 3 ml of sterile air. The liquid and gas were injected into a pre-evacuated 38 ml serum bottle, the liquid acidified with 4 N H_2SO_4 and the evolved CO_2 flushed with N_2 through two scintillation vials in series. Each vial contained 10 ml aqueous counting solution (ACS) fluor and 1 ml Carbo Sorb II for trapping CO_2. The vials were analyzed by liquid scintillation spectrometry (Beckman LS 3801). The amount of radiolabeled recovered as $^{14}CO_2$ was reported as percentage of the amount of radiolabeled added originally.

3.5 Thin layer chromatography (TLC).

Two solvent systems were tested for their ability to separate parent PAH

compounds from metabolites. Solvent A consisted of benzene:acetone:acetic acid (85:15:5). Solvent B consisted of hexane:ether:acetic acid (70:30:2). The separated compounds were visualized by ultra-violet (UV) light using a Chromato-vue Ultraviolet Product Inc. Components appeared as purple spots on a white background.

To determine purity, radiolabeled PAH was applied to silica gel sheets (Kodak Chromogram 13181 with fluorescent indicator) and developed in solvent B. [9-^{14}C]-Phenanthrene and [9-^{14}C]-anthracene were applied in 2 μl portions. The compounds were visualized under UV light and subjected to autoradiography. TLC sheets were placed with Kodak Scientific Imaging film, X-OMAT AR Cat No. 165 1512 (35 x 43 cm) in an autoradiography cassete at -70°C for 3 days. They were developed by RG II Fuji x-ray Film Processor. The original silica gel sheet was cut into strips for each compound and each strip was cut into 1 cm pieces and each piece was added to a scintillation vial containing ACS fluor. The vials were analyzed by liquid scintillation spectrometry (Beckman LS 3801).

Preparative TLC, carried out to purify phenanthrene for further mineralization studies, was performed using 1 mm silica gel sheets precoated with a silica gel containing fluorescent indicator (254 nm) (20 x 20 cm, Si 500F; J. T. Baker Inc., N. J.). Solvent B was used to develop the chromatogram. Bands which fluoresced under short wavelength UV light were scraped off and extracted with dichloromethane, and the silica gel suspension was centrifuged in a clinical centrifuge for 3 min. The supernatant was collected in a dark-glass vial. The solvent was evaporated, the ^{14}C-PAH redissolved in a known volume of dichloromethane and used for mineralization.

3.6 Large-scale production of laccase.

3.6.1 Multiple flask production.

Initially, production of enzyme for purification was done in 2 L flasks containing 1 L of production medium. After inoculation the flasks were incubated at 28°C, and shaken at 200 rpm. Flasks were sampled for laccase activity and

harvested when activity peaked. The medium pH and reducing sugar content were also monitored.

3.6.2 Fermentor production.

A 14 L fermentor containing 10 L of production medium was utilized for large-scale production of laccase. The fermentor was maintained at 28°C and was aerated by forced air at 0.2 v/v/min. The contents were stirred by a marine impeller at 250 rpm. Samples were taken daily and analyzed for enzyme activity, reducing sugar content and pH.

3.7 Purification of laccase

Fungal mycelium and residual bran flakes were removed from the culture supernatant by filtration through nylon prior to centrifugation at 6000 rpm for 30 min. The clarified medium was frozen for 3 d and thawed. The precipitate which formed was removed by centrifugation at 6000 rpm for 30 min in a centrifuge. All purification steps were carried out at 4°C. After concentration of the supernatant by ultrafiltration using an Amicon filtration unit with a PM-10 membrane, the sample was centrifuged at 10000 x g for 30 min to remove any particulate matter. The concentrate was purified by passage through a 500 ml anion exchange column (DE-52) pre-equilibrated with 20 mM phosphate buffer, pH 6.0 and eluted with a linear gradient of 0 to 0.4 M NaCl in 20 mM phosphate buffer. Fractions containing laccase activity were pooled and dialyzed against 20 mM phosphate buffer and reconcentrated by ultrafiltration and the process repeated as before in a 200 ml DE-52 column. Again, fractions containing laccase activity were pooled, dialyzed and concentrated. The concentrate was passed through a gel filtration column (Sephadex G100) pre-equilibrated with 0.1 M KCl in 20 mM phosphate buffer. The fractions containing laccase activity were pooled and concentrated. The enzyme was finally purified by Fast Protein Liquid Chromatography (FPLC) using a Mono Q or High Q column.

3.8 Polyacrylamide gel electrophoresis.

3.8.1 Denaturing gel electrophoresis.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was used to follow the purification process. A 10% polyacrylamide – 0.1% SDS gel was run at 200 V for 40 min, according to the method of Laemmli. The sample buffer was 62.5 mM tris-HCl, pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue. β-Mercaptoethanol (50 μl) was added to 950 μl of sample buffer. The sample was diluted 1:2 with sample buffer. The gel was fixed in 40% methanol for 30 min and stained in 0.01% Coomassie Brilliant Blue R-250 in 40% methanol for 45 min. The gel was destained 3 hours, with periodic changes in destaining solution (40% methanol, 10% acetic acid and 50% water).

Low and high molecular weight standards purchased from Bio-Rad. The low molecular weight markers were (Da) bovine serum albumin, 66000, porcine heart fumarase, 48500, carbonic anhydrase, 29000, β-lactoglobulin, 18400, and α-lactalbumin, 14200. The high molecular weight markers include β-galactosidase, 116000, phosphorylase b, 97400, bovine serum albumin, 66000, porcine heart fumarase, 48500, carbonic anhydrase, 29000.

3.8.2 Non-denaturing gel electrophoresis.

Precast Bio-Rad mini gels were run under non-denaturing conditions. The gel was run for 35 min at 200 V in the cold room. Running buffer contained (g/l): 3.0 g of Tris base and 14.4 g of glycine, pH 8. Sample buffer contained: 4.9 ml dH_2O, 1.0 ml 0.5 M Tris-HCl (pH 6.8), 2.0 ml glycerol and 1 ml 1% bromophenol blue. Duplicate lanes were run to test two potential substrates for activity staining. Each lane was loaded with 10 μg of protein. After completion of the run, the gel was cut in two and each half was submerged in substrate, *o*-dianisidine or ABTS, 1 mM in 0.1 M sodium acetate buffer, pH 4.0.

3.9 Characterization of purified laccase.

3.9.1 Chemical characteristics.

Matrix assisted laser desorption ionization (MALDI) mass spectra were recorded on an HP time of flight (TOF) instrument. Protein samples of 2.5 mg/ml and sinappinic acid, as the matrix, were loaded onto the sample plate and the spectra were acquired using bovine serum albumin for external calibration. Mass spectra were performed by the laboratory of Dr Liang Li, Department of Chemistry, University of Alberta.

For the spectroscopic characterization of the Cu (II) centers of laccase, 0.2 mg of the enzyme dissolved in phosphate buffer, pH 6, was used. Spectroscopic measurements were carried out using an Unicam UV-Vis 8700 Series spectrophotometer. Scanning from 800 nm to 200 nm. Copper content was measured by the method of Felsenfeld (1960), using 2,2'-biquinoline in glacial acetic acid and ascorbic acid as the reducing agent.

Carbohydrate content was determined by the phenol method of Dubois et al. (1956) using glucose as a standard.

N- terminal amino acid sequencing was performed by the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta, Canada.

3.9.2 Physical characteristics.

3.9.2.1 Study of pH optimum and stability.

To determine the optimum pH for the enzyme, activity was monitored at pH values ranging from 2.5 to 7.0 using ABTS as substrate. To determine the pH stability of the enzyme, the enzyme was diluted to the pH value tested ranging from pH 3 to 10 at 25°C. Activity was measured by oxidation of ABTS in 0.1 mM NaOAc buffer, pH 4.0, at 30°C.

3.9.2.2 Study of temperature optimum and stability.

To determine the temperature of highest activity for laccase, activity was measured from 25°C to 85°C, in 10 °C increments using ABTS as substrate. To estimate enzyme thermal stability, activity was measured under standard

conditions at 30°C after preincubation at 25°C to 85°C, again following the oxidation of ABTS.

3.9.2.3 Substrate specificity.

Spectrophotometric measurement of substrate oxidation of laccase was carried out at 30°C in a 1 ml reaction volume containing the test substrate at 0.5 to 5 mM in 0.1 M sodium acetate, pH 4.0. The substrates are tabulated with the wavelength at which they were tested and the extinction coefficient. Syringaldazine was first dissolved in ethanol, then brought to 1 mM with the addition of 0.1 M sodium acetate, pH 4. Other methods of measurement including O_2 consumption were not used.

Table 3. Potential substrates for laccase.

Substrate	Wavelength (nm)	Extinction Coefficient (M^{-1} cm^{-1})
Vanillin	355	1930
Vanillic acid	316	2340
ABTS[a]	436	29300
HBT	343	2530
2,6-dimethoxyphenol[b]	470	35645
Catechol[b]	450	2211
Syringaldazine[c]	530	65000
Syringaldehyde	250 270	1200
p-methoxyphenol[d]	470 253	6740 4990
4-aminophenol[d]	246	15627
Tyrosine	280	N/A

[a] Wolfenden and Wilson, 1982;[b] Eggert et al., 1996;[c] Xu, 1996;[d] Munoz et al., 1997

3.9.2.4 Enzyme inhibition.

The potential inhibition of ABTS oxidation was monitored in the presence of 1 mM ABTS under standard conditions. The inhibitors tested were L-cysteine, EDTA, thioglycollic acid, sodium azide, phenanthroline and dithiothreitol.

3.10 Chemical modification of laccase.

3.10.1 Polyethylene glycolation of free amino groups on laccase.

A five-fold excess of methoxypolyethylene glycol activated with cyanuric chloride (PEG-CN, mol wt 5000) was utilized. Laccase (2 mg protein) and 5 mg of PEG-CN were added to 4 ml of 40 mM borate buffer, pH 10. The reaction took place at room temperature for 2 h. It was then diluted ten-fold and concentrated by ultrafiltration (Amicon PM-10), three times. Each time the preparation was concentrated to 4 ml (Vazquez-Duhalt et al., 1995).

To modify approximately half the amount of free amino groups the reaction was performed with only a 2.5-fold excess of PEG-CN and was reacted for 1 h at room temperature, as determined empirically.

3.10.2 Methylation of free carboxyl groups on laccase.

Laccase (6 mg protein) was dried under vacuum, dissolved in 2 ml of N'N-dimethylformamide and 2 ml of boron trifluoride (BF_3) in methanol reagent was added. The reaction took place at room temperature for 12 h. The reaction mixture was diluted to 40 ml with phosphate buffer, pH 6.0 and filtered through a 0.45 μm nylon membrane. The filtrate was then dialyzed and concentrated by ultrafiltration as above (Tinoco and Vazquez-Duhalt, 1998).

3.10.3 Characterization of chemically modified laccases.

Spectra, activity, stability and substrate specificity of chemically modified laccase were determined as described for the unmodified enzyme. From 0% to 60% acetonitrile was added to the assay mixture or dilution buffer where appropriate.

3.10.4 Oxidation of PAH by laccase.

3.10.4.1 *In vitro* oxidation of PAH by laccase.

Laccase mediated oxidation of PAHs was determined by incubating a mixture of individual PAHs (20 μM, final concentration) in 15% acetonitrile in 0.1 M acetate buffer, pH 4, with 5 units of laccase, PEG-laccase, ½-PEG-laccase or Met-laccase in a 100 μl reaction volume. Mediating substrates were added to the mixture to a final concentration of 1mM, including ABTS and HBT. The assay was started by the addition of the enzyme and was terminated by the addition of acetonitrile to a final concentration of 60%. Boiled enzyme controls showed no activity. After centrifugation, 10 μl samples were analyzed by high performance liquid chromatography (HPLC) by using a C18 reverse-phase column (Brownlee Labs Inc., Santa Clara) and isocratic elution with acetonitrile:water (60:40). Peak areas were calculated and the first order oxidation reaction rates were plotted by fitting the data to the equation $A_o = A_t e^{-kt}$ (Pickard et al., 1999). A_o represent the initial concentration of the compound being tested. A_t represents the final concentration of the compound being tested.

3.10.4.2 High performance liquid chromatography.

Analyses by HPLC were carried out on a Waters (WISP) model 712 Automatic Injector and a Model 486 Tunable Absorbance Detector, (Waters Scientific Co., Mississauga). The integrator was an HP model 3392A. An analytical reverse phase column (RP-18, 100 x 4.6 ll, Brownlee Labs Inc.) was used. The eluant was monitored at wavelengths from 225 to 255 nm. The mobile phase was 60:40, acetonitrile:water (Pickard et al., 1999).

4. Results and Discussion

4.1 Design of production medium.

Culture conditions and medium composition can play a major role in the level of enzyme production. Studies were conducted to design a medium supporting maximum enzyme production by *C. gallica*. Enzyme production has been shown to be increased by the presence of a natural lignin source. Ground cereal bran was investigated as a carbon source for laccase production (Pickard et al., 1999). As seen in Figure 10, cereal bran was effective in supporting laccase production. This study compared cereal bran flakes, wheat bran, oat bran and pine sawdust as lignin sources. Both bran flakes and oat bran supported good laccase production. Production started at 2-4 days and maximum activity was reached shortly. The maximum activity ranged from 8 to 12 units/ml. After a longer delay wheat bran also supported enzyme production up to 6 units/ml. The GMY medium control and sawdust did not support enzyme production. Of the lignin sources tested, bran flakes were the best carbon source for laccase production (Pickard et al., 1999).

Bran flakes, the best enhancer of laccase production, contains potential carbon and energy sources, other than lignin, for growth. It contains glucose and starch for initial rapid growth, then, upon exhaustion, the fungus has enough biomass to produce sufficient enzyme to break down lignin as a carbon and energy source in a relatively short period of time. Other lignin sources, such as wheat bran, do not contain additional sugar or starch, so the amount of biomass depends solely on the breakdown of lignin. There is an initial lag period to become accustomed to the conditions of starvation from a nutrient rich medium, GMY, for production of laccase. There is no alternative source for rapid growth, so initial growth is slower. For this reason laccase production was not as fast in the presence of wheat bran as when bran flakes were present. In the presence of pine sawdust there was no enzyme production because *C. gallica* did not grow in the flasks containing pine sawdust. Upon visual inspection, it was clear that

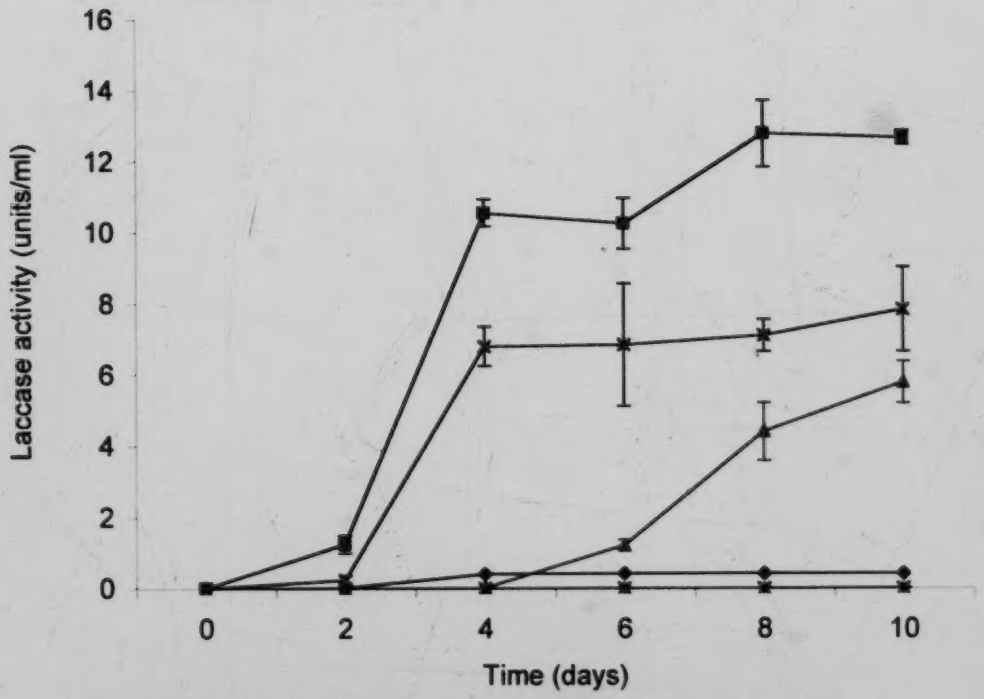


Figure 10. Ground Cereal Bran in phosphate buffer supports laccase production by *C. gallica* (error bar represent samples from triplicate flasks)

GMY — Bran Flakes — Wheat Bran — Oat Bran — Sawdust

there was no growth of *C. gallica* as no pellets were formed and the medium remained clear. White rot fungi can be specific for the type of wood (lignin) they can use and pine sawdust is possibly a type that cannot be used by *C. gallica*. Oat bran did support good production of laccase. There was no sugar added in the oat bran but the lag period for laccase production is similar to bran flakes. Oat bran naturally may have more easily accessible sugars present in its composition than wheat bran, which the fungus will grow on initially to obtain biomass for increased laccase production. It is for this reason that more enzyme may have been produced when oat bran was available to the fungus than wheat bran. It is clear from the study that the best source of lignin to enhance laccase production by *C. gallica* is bran flakes.

Previous studies have shown that several fungi, including *C. gallica*, have increased laccase production when grown in solid-state fermentation with whole oats (Rodriquez et al., 1999). A wheat bran medium was shown to produce higher levels of laccase production in *Ceriporiopsis subvermispora*, at 3.85 units/ml as compared to rich medium at 2.12 units/ml (Salas et al., 1995). It has also been shown that laccase production in several fungi can be enhanced with cotton stalk extract (Ardon et al., 1996, 1998) and chopped barley straw (Rodriguez et al., 1997). Growth of *T. versicolor* on both wheat straw and beech wood led to an increase as high as 3.5 fold in extracellular laccase activity, in comparison with growth on glucose (Schlosser et al., 1997). Production of enzyme by *C. subvermispora* was enhanced in wheat bran medium as opposed to a minimal medium supplemented with Cu or chemical inducers such as 2,5-xylidine (Fukushima and Kirk, 1995). Enzyme production by a variety of fungi has been shown in several studies to be enhanced by the presence of a natural lignin source. In this study ground cereal bran was investigated to provide a similar carbon source. Cereal bran is an effective lignin source to support laccase production. Of the lignin sources tested, bran flakes was the best carbon source for laccase production, able to produce up to 15 units/ml of laccase activity, one of the highest levels reported for this fungus.

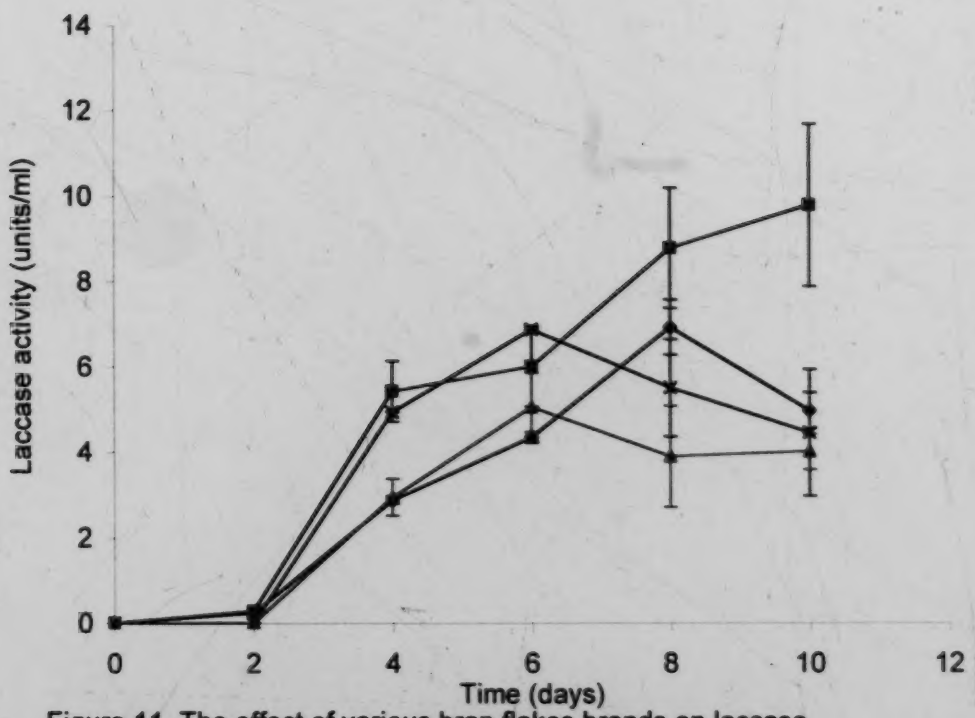


Figure 11. The effect of various bran flakes brands on laccase production by *C. gallica* (error bars represent samples from triplicate flasks).

Different commercial sources of bran flakes exhibit differences in taste, color, sweetness and additions. Therefore several brands of bran flakes were compared for their ability to support laccase production. However, as shown in Figure 11 there is little difference in laccase production between the various products. Each brand of bran flakes supported laccase production. The No Name product from Superstore did seem to enhance laccase production more than other brands. There was a significant difference in activity on day 10 over the other brands. There was also significant variation within the triplicate flasks in activity of the No Name bran flakes compared to Kellogg's bran flakes. The No Name brand tends to vary in composition from lot to lot because the manufacturer varies, whereas Kellogg's, the next best brand, is consistently produced. For practical reasons all further studies were carried out with Kellogg's bran flakes.

The composition of the bran flakes did not have an effect on laccase production. The contents of the bran flakes are similar. There is minor variation in some of the vitamins and cofactors present and in the types of sugar present, but these do not correlate with an increase or decrease in enzyme production. It is postulated that the bran flakes with the most readily utilizable carbon source for best initial biomass production should produce the most enzyme, but minor differences in composition do not have an effect on laccase production.

The carbon source level in the medium was the next parameter studied and is an important consideration for the cost effectiveness of the medium. Laccase production increased with the amount of bran flakes present in the medium as seen in Figure 12. At all levels of bran flakes, enzyme production leveled off between 7 and 10 days. At the end of growth, the media containing 1% and 2% bran flakes were clear, as no bran flakes remained undigested in the flasks. At 3% bran flakes, the enzyme production also leveled off and a little bran remained. At 4% bran flakes, the enzyme level was increasing as the medium was still turbid with undigested bran flakes even at 10 days, but laccase production had slowed. As there is more carbon source present, it would be

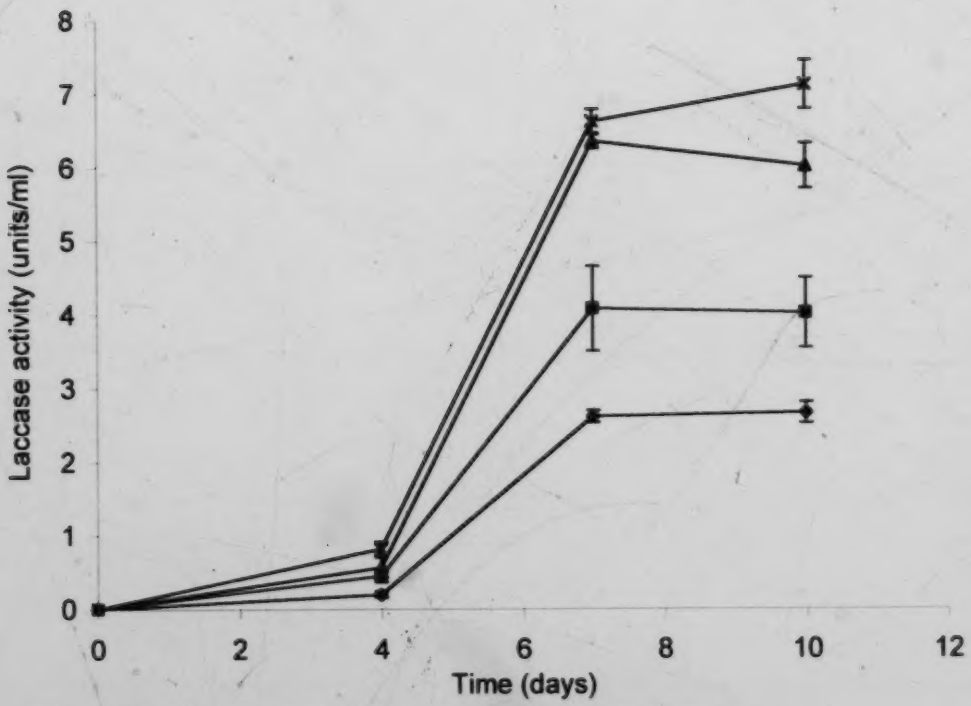


Figure 12. The effect of medium carbon source level on laccase production by *C. gallica* (error bars represent samples from triplicate flasks).

logical that there would be more biomass present to produce more enzyme. There is an upper limit to this prediction. A compromise must be met between the growth of the fungus and viscosity of the medium. If the medium is too viscous, there is potential for loss of enzyme due to inhibited fungal growth. As well, viscous medium is difficult to handle as aeration and other mass transfer limitations can become a problem. The medium was very viscous with 4% bran flakes but was manageable with 3% bran flakes. For practical reasons, 3% solids was chosen as the best level for laccase production.

Another important parameter is the effect of the initial pH of the medium. The pH of the medium can have a marked effect on the growth of the fungus. Therefore, an experiment was conducted using medium buffered over a range of initial pH values. The initial pH of the 60 mM phosphate buffer in the bran suspension has little effect on laccase production over the range of pH 4-6 as seen in Figure 13. After 10 days, laccase production was similar in all cultures. One exception was at pH 6.5, where enzyme production was slightly higher at the end of the experiment. All other curves appeared similar. The pH of the medium did not change as the experiment progressed. For this reason, phosphate buffer at pH 6.5 was chosen as the best buffer for enzyme production.

Microorganisms tend to have a certain pH range in which they grow the best. This is usually around neutrality but fungi tend to prefer growth in slightly more acidic environments than most bacteria. This study was designed to look at the growth of the fungus balanced against the activity and stability of the enzyme. The enzyme also has an optimal range in which it is the most active. For laccase, the optimal range is around pH 4. If the enzyme is most active around pH 4 but is most stable around pH 6 – 6.5, then this may be the reason for increased levels observed at more neutral pH: the enzyme is being inactivated by acidity before it can be measured at lower pH. If the fungal growth is greater at higher pH, there will be more laccase content at that pH, simply due to more fungal biomass. However, visual inspection of the flasks indicated that fungal growth was relatively equal between the flasks. Looking at the results it is

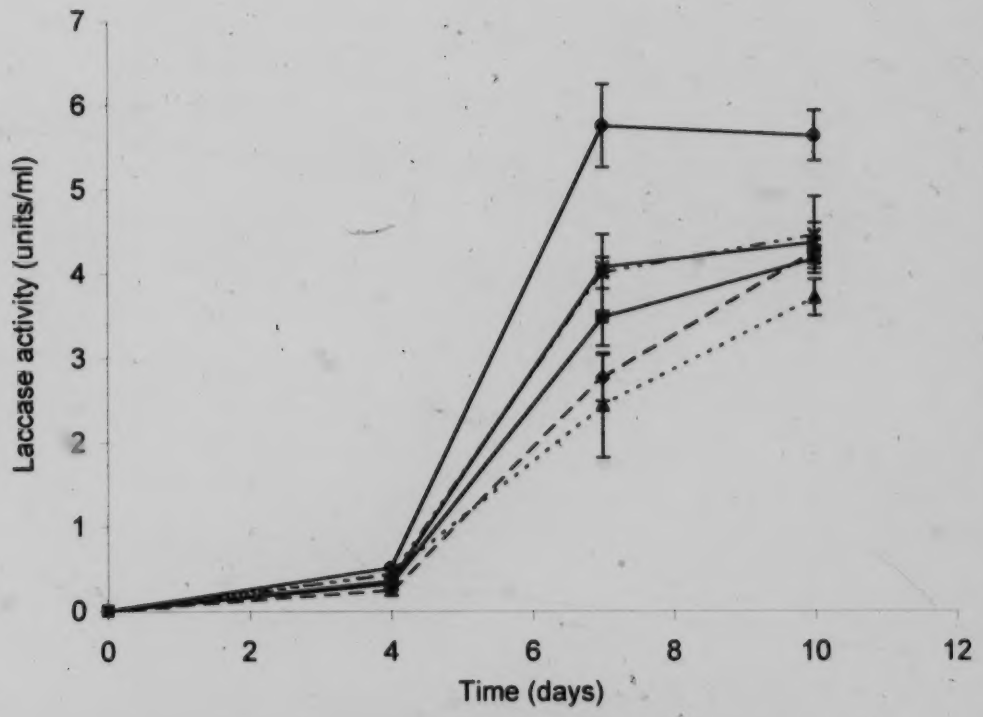


Figure 13. The effect of medium pH on laccase production by *C. gallica* (error bars represent samples from triplicate flasks)

– ◆ – pH 4.0 —■— pH 4.5 · · ▲ · · pH 5.0 —✱— pH 5.5

– ✱ – pH 6.0 —●— pH 6.5

clear that the pH does not have an impact on enzyme production via growth of the organism and does not have an impact on the activity of the enzyme due to inactivation at a certain pH. There was no change in the pH of the medium, so the fungus was not changing the pH to produce more laccase. Variations in the flasks may be natural variation.

Oxygen can be an important factor in the growth of microorganisms and therefore enzyme production. Figure 14 shows that even at the highest medium volume in the flask enzyme production proceeded equally between the flasks, as laccase content was consistent in all of the flasks. Partial air volume does not have an effect on laccase production by *C. gallica* in flasks. The amount of medium in the flask can be an important parameter due to the necessity of oxygen for fungal growth. One of the main problems associated with oxygen is the mass transfer of oxygen throughout the medium. Shaking a flask can aid in the transfer of oxygen to all of the medium but as the volume of the medium increases in a flask, transfer of oxygen becomes more difficult. However even at 50% medium volume, aeration by shaking is sufficient for growth and production of laccase by *C. gallica*.

This lack of relationship between laccases activity and aeration has been found in the literature for other laccase producers. There was no clear relationship observed between laccase and oxygen pressure in *Phanerochaete flavido-alba* (Perez et al., 1996). Ruttiman-Johnson et al. (1994) also could not establish a clear correlation between these two parameters in other white rot fungi. As this enzyme is an oxidase, lack of oxygen can restrict the activity of the laccase, therefore making it unable to degrade lignin to make it available as a carbon source but it does not appear to be a limiting factor under these conditions. According to these data, up to half of the capacity of the flask can be utilized but upon autoclaving the medium overflows the flasks. For this reason, it was decided to fill flasks with 0.4 of production medium of the total volume.

A problem with an insoluble medium is the difficulty of sterilizing particulates and the resulting contamination. It was thought that solid bran flakes

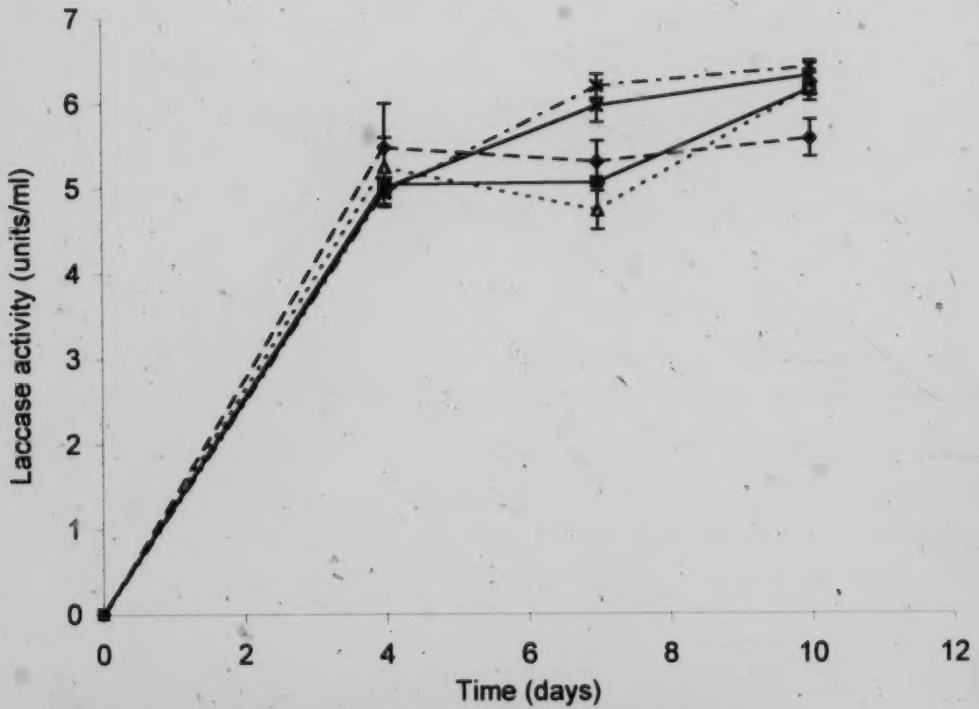


Figure 14. The effect of medium volume in 500ml flasks on laccase production by *C. gallica* (error bars represent samples from triplicate flasks)

– ◆ – 50 ml —■— 100 ml · · ▲ · · 150 ml —✕— 200 ml – ✱ · 250 ml

might not be needed to support enzyme production, but that enough lignin was solubilized by heating to support laccase production. However, the fungus did not produce significant levels of enzyme when grown on soluble lignin, which is the centrifuged supernatant from autoclaved bran flakes. Figure 15 shows that enzyme production was significantly lower when insoluble material was removed by centrifugation prior to inoculation. Presumably, the amount of soluble lignin was low and rapidly used by the fungus. Fungal growth also was significantly less in the flasks when particulates were removed. This is mainly due to less available carbon and energy source for growth, resulting in lower laccase levels. It is clear from this experiment that the particulates must be present for enhanced laccase production.

In previous studies, it has been postulated that it is a solubilized component of lignin that has been responsible for enhanced production of laccase grown on lignin sources. This is because lignin model compounds, such as veratryl alcohol, were effective in increasing laccase production. As well, the mycelium was not in contact with the lignin source. This is true for *T. versicolor* where beechwood and wheat straw were not intimately associated with mycelium of the fungus (Schlosser et al., 1997). It was seen in this study that the bran flakes were attached to the fungus, so it is unlikely that a solubilized product was solely responsible for enhanced enzyme production. As biomass could not be separated from bran flakes, it was difficult to determine the extent of growth to see whether enzyme production was directly related to biomass, or if the activity was enhanced by solubilized lignin. It seems clear by this study there was enzyme production in solubilized lignin cultures but that the best enzyme production occurred when particulate lignin source was available.

Synthesizing the results from these experiments, the production medium was designed to contain 3% Kellogg's bran flakes in 60 mM potassium phosphate buffer at pH 6.5, at 2/5 the volume of the reactor vessel with the bran flakes present to enhance laccase production. For initial growth in the production medium, the inoculum from GMY rich medium provides adequate nutrients for

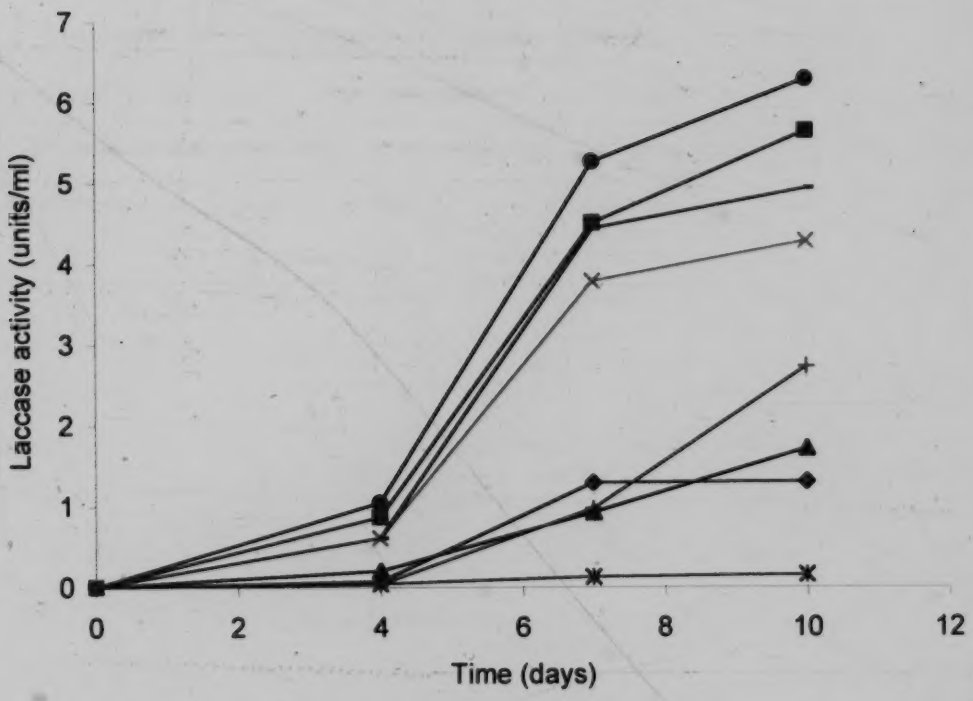


Figure 15. Production laccase on solubilized lignin by *C. gallica*

Kelloggs C
Kelloggs UC
Safeway C
Safeway UC
No Name C
No Name UC
Western Family C
Western Family UC

initial growth. After determining the conditions for best production of laccase enzyme, it was thought that this could be exploited to determine if *C. gallica* will mineralize PAHs *in vivo* and if this is due to laccase.

4.2 PAH mineralization by *C. gallica*.

4.2.1 Mineralization in bran flakes medium.

One of the main objectives of this project was to look at the degradation of PAHs. Quinones and quinols have been reported as metabolites of fungal PAH degradation. Ring fission reactions during subsequent metabolization of quinones and quinols make it possible for complete mineralization of PAHs to CO_2 and water. However, numerous experiments performed with ^{14}C-labelled compounds have demonstrated relatively low mineralization rates (Majcherczyk, 1998). Several PAHs were examined for their ability to be mineralized by *C. gallica* including anthracene, phenanthrene and naphthalene. Each of these was tested for mineralization by the fungus under different conditions. GMY medium with killed cells and live cells was compared to bran flakes medium with killed and live cells. As seen in Figures 16-18 there was more mineralization of all three PAHs in the bran flakes medium with living cells over all other conditions tested. For naphthalene, there was 5% mineralization at day 15 in bran flakes medium with culture whereas there was at maximum 3% in all other conditions. For phenanthrene there was 6% mineralization in bran flakes medium with culture but less than 3% mineralization in all other conditions tested. For anthracene there was over 2% mineralization in bran flakes medium with culture but less than 1% in all other conditions.

The GMY medium, as seen in Figure 10, does not support high laccase content whereas the bran flakes medium does produce high laccase content. If there is higher mineralization in the bran flakes medium than in the GMY medium this may indicate a relationship between PAH mineralization and laccase production. As seen in Figures 16 –18 there is some mineralization of all three PAHs in the bran flakes medium with living cells. In comparing the PAHs, as seen in Figure 19, naphthalene and phenanthrene are the better substrates.

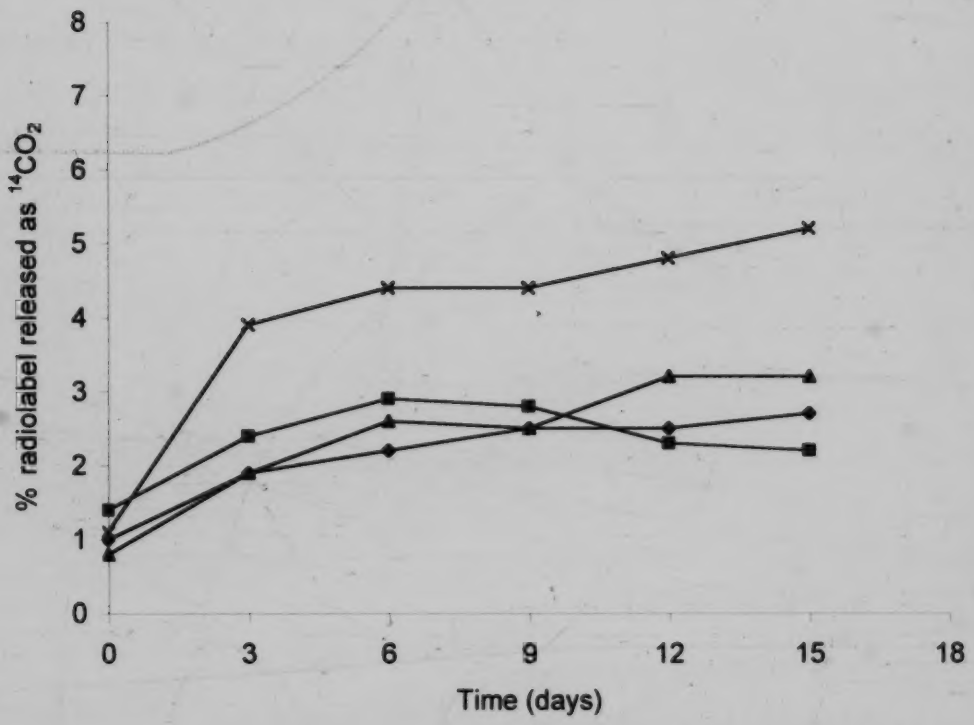


Figure 16. Mineralization of [1-^{14}C]-naphthalene by *C. gallica*
—◆— GMY killed —■— GMY live —▲— KBF killed —✕— KBF live

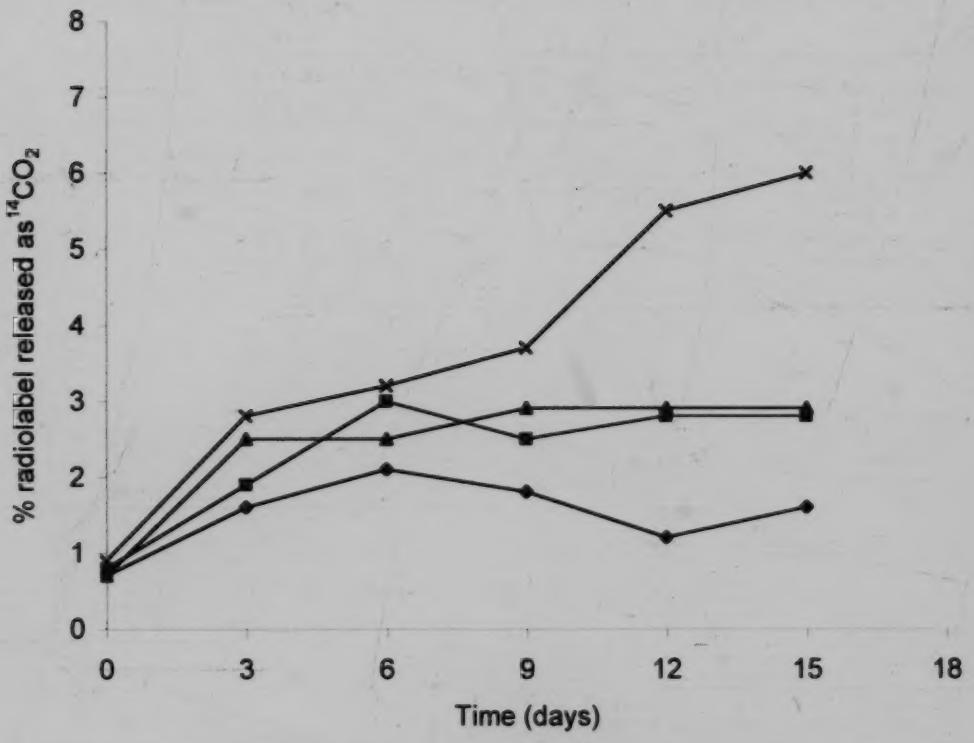


Figure 17. Mineralization of [9-^{14}C]-phenanthrene by *C. gallica*
—◆— GMY killed —■— GMY live —▲— KBF killed —✕— KBF live

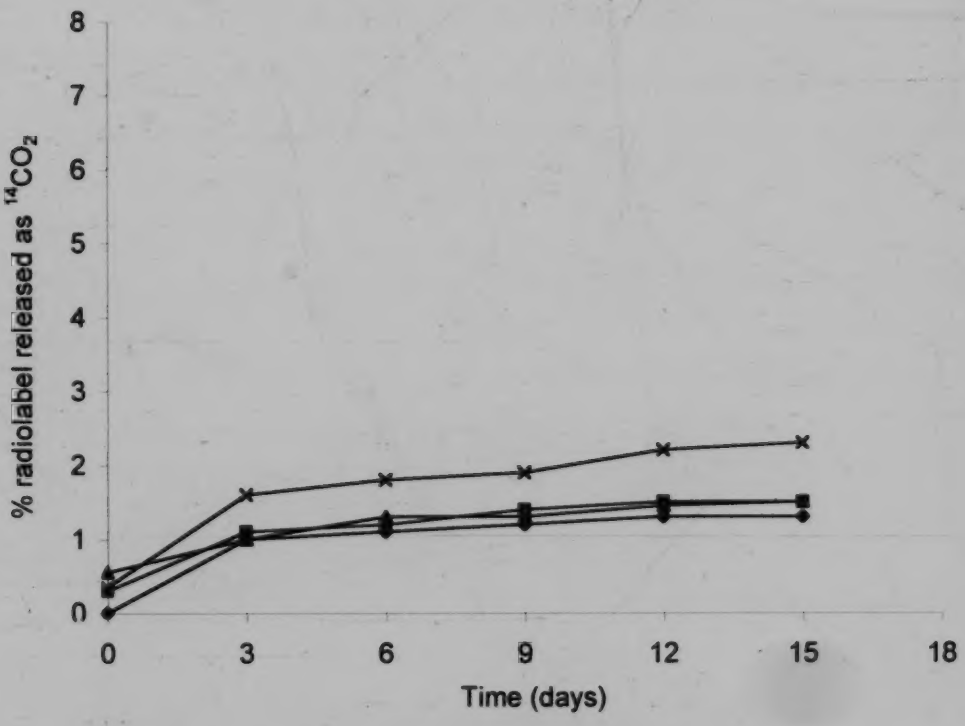


Figure 18. Mineralization of [9-^{14}C]-anthracene by *C. gallica*

GMY killed GMY live KBF killed KBF live

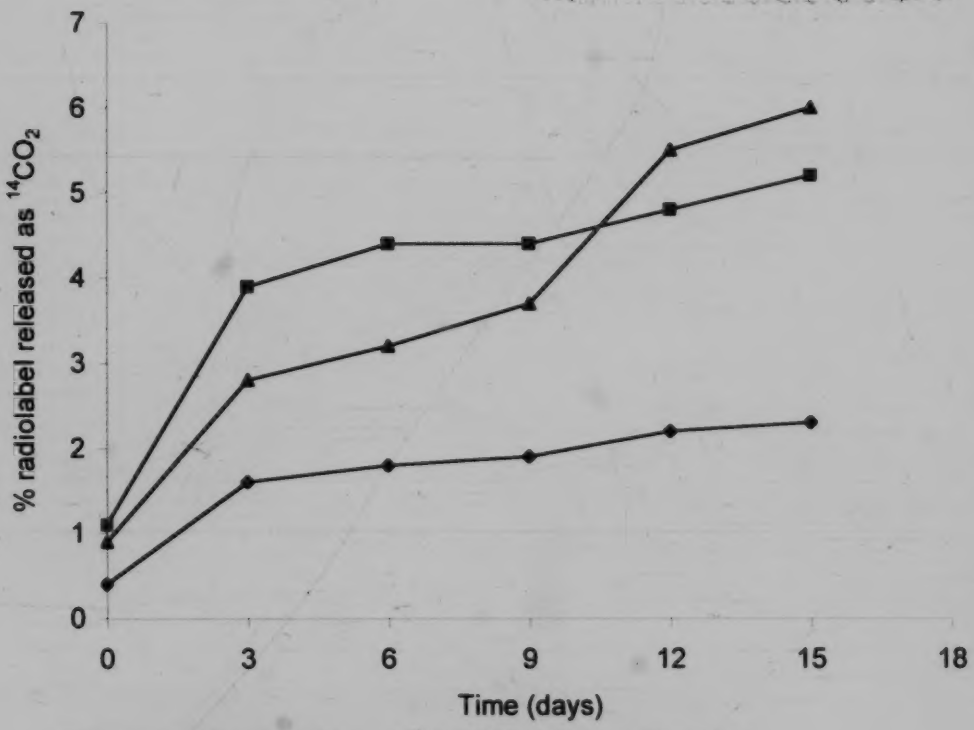


Figure 19. Mineralization of ^{14}C-PAHs by live cultures of *C. gallica* grown in KBF.

—◆— Anthracene —■— Naphthalene —▲— Phenanthrene

4.2.2 Mineralization in the presence of mediating substrates.

It has been shown that the addition of an artificial laccase substrate can enhance lignin degradation by acting as a mediating substrate, enabling the oxidation of nonphenolic compounds that are not laccase substrates on their own. It may be possible that the addition of a mediating substrate would enhance mineralization of PAHs by *C. gallica*. When PAHs were incubated with the fungus in the presence of mediating substrates, there was more mineralization of the PAHs compared to cultures without mediating substrates. For naphthalene, there was 6% mineralization in the presence of ABTS, 6% mineralization in the presence of HBT and 4% mineralization without the presence of mediating substrates on day 10. Upon the addition of another mediating substrate to the mixture, there was a further increase in mineralization. The addition of HBT to the ABTS culture increased mineralization to 12% but the addition of ABTS to the HBT culture increased mineralization to 8% and the culture without mediating substrates remained at 4% mineralization on day 20.

Similar trends were observed when anthracene was tested. In the presence of ABTS, there was 2.5% mineralization of anthracene. In the presence of HBT there was 3% mineralization of anthracene and without mediating substrates there was 2% mineralization of anthracene. Upon addition of a second mediating substrate to the cultures, there was a further increase in mineralization. When HBT was added to the culture with ABTS there was an increase in mineralization to 5%. The addition of ABTS to the culture supplemented with HBT did not increase mineralization.

For phenanthrene, there was also an increase in the amount of mineralization when mediating substrates were present. In the presence of ABTS there was 4% mineralization of phenanthrene. In the presence of HBT there was 4.5% mineralization of phenanthrene and without a mediating substrate there was 2.5% mineralization. Upon addition of a second mediating substrate to the cultures there was a slight increase in mineralization. The addition of HBT to ABTS increased mineralization to 5%. The addition of ABTS

to HBT culture increased mineralization to 5.5%.

The addition of mediating substrates enhances the mineralization of PAHs. Specifically, the addition of HBT enhances mineralization better than ABTS in most cases. This may be due to the nature of the radical formed by the mediating substrates. HBT forms a nitroxyradical that is more reactive and less stable than the cation radical formed from ABTS. The nitroxyradical is a more potent electrophile that is able to initiate attack of aromatic compounds better than the radical of ABTS. This is exemplified not only upon addition of a single mediating substrate, but also upon the addition of a second mediating substrate. The addition of HBT to ABTS containing cultures increased mineralization more than the addition of ABTS to HBT containing cultures. It is clear that mineralization of PAHs does occur and that mediating substrates do accelerate the process, again leading to the conclusion that laccase is involved in PAH degradation. However, the amount of mineralization in these experiments is low and may not be significant in the environment.

4.2.3 Thin layer chromatography for purification of ^{14}C-PAH.

As the mineralization of these PAHs is very low there is the possibility that the mineralization detected is not in fact from the PAH but from a contaminant in the solution. Phenanthrene and other PAHs are photosensitive therefore upon exposure to light the compound is degraded. Other compounds in the PAH solutions were detected by TLC. Two solvents were tested and shown to produce different migration of the PAH and their photo degraded chemicals, quinones. Visualization under UV light showed that solvent A did not separate anthracene from anthraquinone very well as seen in the R_f values in Table 4. Phenanthrene and phenanthrenequinone migrated to sufficiently different locations. Solvent B resolved phenanthrene and phenanthrenequinone but also resolved anthracene and anthraquinone sufficiently for detection as seen in the R_f values in Table 4. Since the parent compounds could be separated from primary degradation products, solvent B was employed to purify the PAHs.

Table 4. Retention factors of nonpolar compounds on TLC.

Compounds	Solvent A R_f	Solvent B R_f
Phenanthrene	0.78	0.68
Phenanthrenequinone	0.28	0.15
Anthracene	0.78	0.72
Anthraquinone	0.74	0.48

Solvent A: benzene:acetone:acetic acid (85:15:5)
Solvent B: hexane:ether:acetic acid (70:30:2)

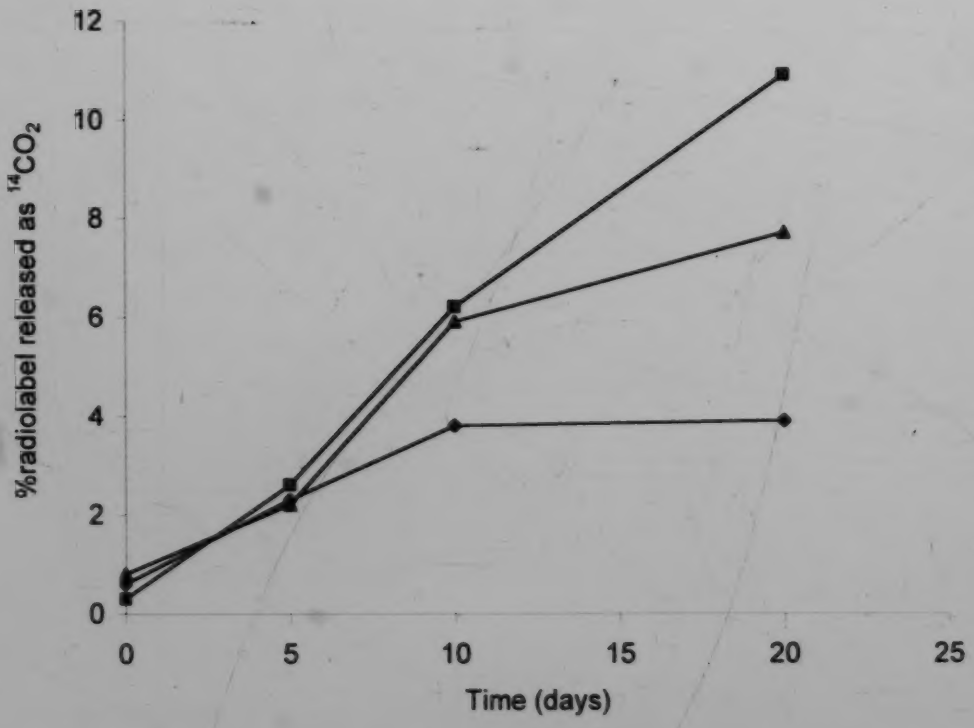


Figure 20. Mineralization of [1-^{14}C]-naphthalene by *C. gallica* in the presence of mediators —◆— no addition —■— ABTS —▲— HBT

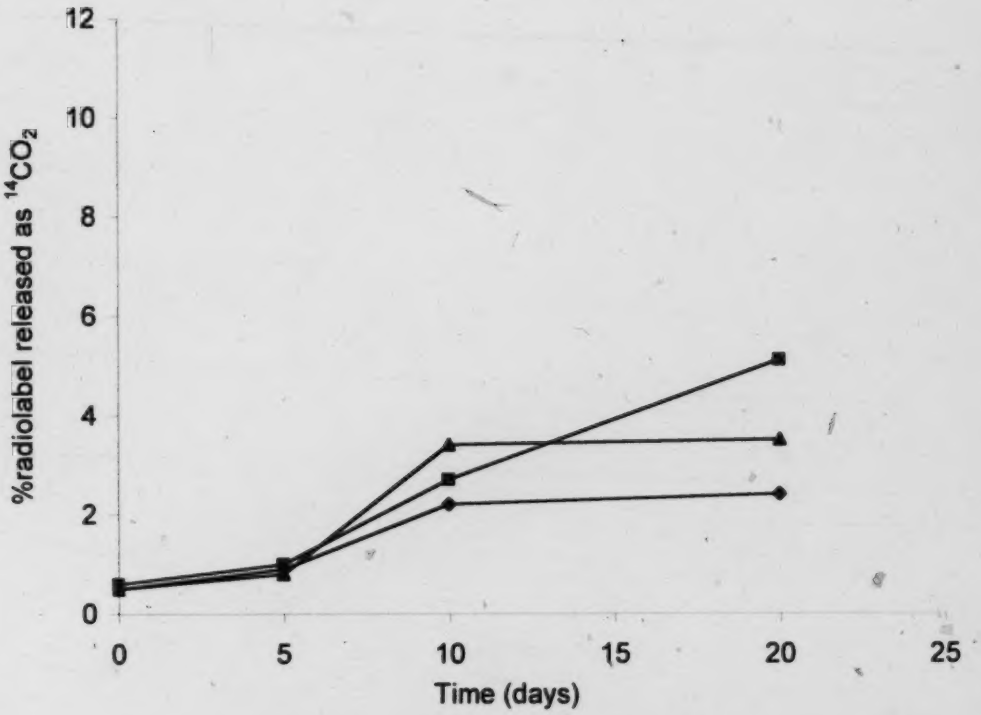


Figure 21. Mineralization of [9-^{14}C]-anthracene by *C. gallica* in the presence of mediators —◆— no addition —■— ABTS —▲— HBT

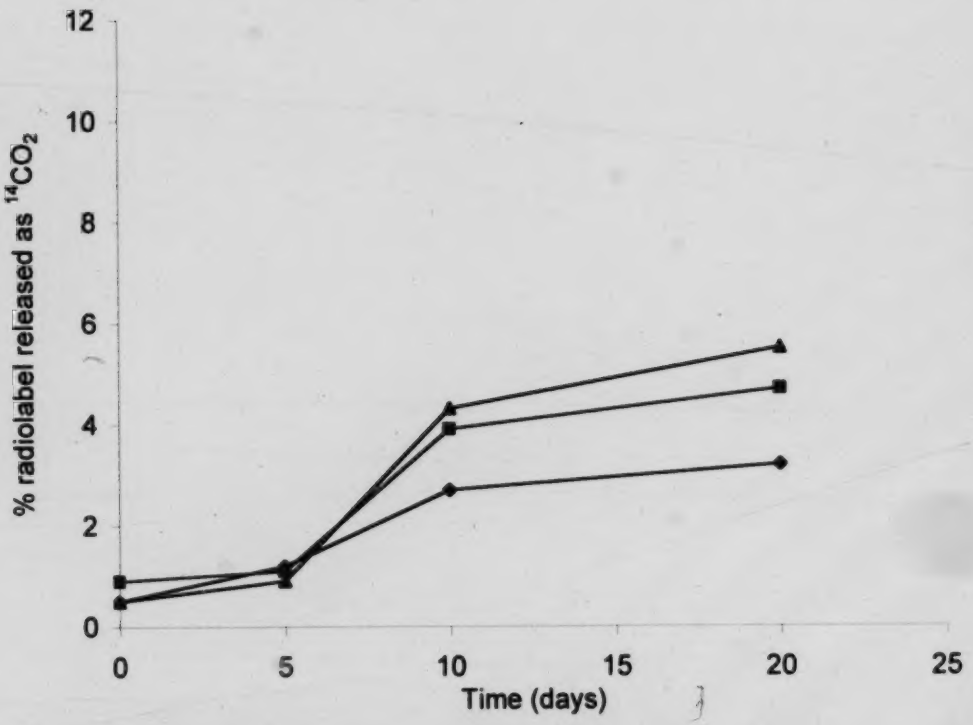


Figure 22. Mineralization of [9-^{14}C]-phenanthrene by *C. gallica* in the presence of mediators —◆— no addition —■— ABTS —▲— HBT

Radioloabelled compounds were found to be contaminated. Phenanthrene was found to be 98% pure and anthracene was found to be 90% pure. Naphthalene was volatile and could not be separated by TLC due to loss of compounds resulting in low recovery. The PAHs were purified by TLC and it was determined that the amount of mineralization in phenanthrene with mediating substrates ABTS and HBT was the only amount that was significant compared to impurities. Phenanthrene has 2% contamination but mineralization was at 6% with unpurified phenanthrene. Phenanthrene was purified by TLC prior to use as a mineralization substrate at a volumetric activity of 1500 dpm/μl. Anthracene mineralization was negligable. It was found to be 90% pure and at 2% mineralization it was unclear whether anthracene or a contaminant was being degraded.

4.2.4 Mineralization of purified phenanthrene.

Purified phenanthrene was tested for its ability to be mineralized by *C. gallica*, *in vivo*. The killed cell control did not produce $^{14}CO_2$ until day 35 when the culture was discovered to be contaminated. The flask with *C. gallica* in bran flakes first showed signs of mineralization between 20 d and 35 d. There was 9% mineralization of phenanthrene from this culture. With the addition of mediating substrates, ABTS and HBT, there was a significant increase in mineralization to 17.5% mineralization of phenanthrene by day 40, as seen in Figure 23.

Our studies have confirmed that *C. gallica* is able to mineralize [^{14}C]phenanthrene in semi solid bran cultures. *In vivo* mineralization of PAHs has been previously shown by WRF. *T. versicolor* was shown to mineralize phenanthrene to 15.5% in 63 days growing on a straw medium (Sack et al., 1997). *C. gallica* is shown here to degrade phenanthrene to 17.5% in 54 days growing on bran flakes medium amended with ABTS and HBT. There appears to be slightly higher mineralization in a shorter period of time. Other reports of phenanthrene mineralization include *P. chrysosporium*, which is able to produce to 7.7% $^{14}CO_2$ in 27 days and 1.3% $^{14}CO_2$ in 70 days (Bumpus, 1989; Morgan et

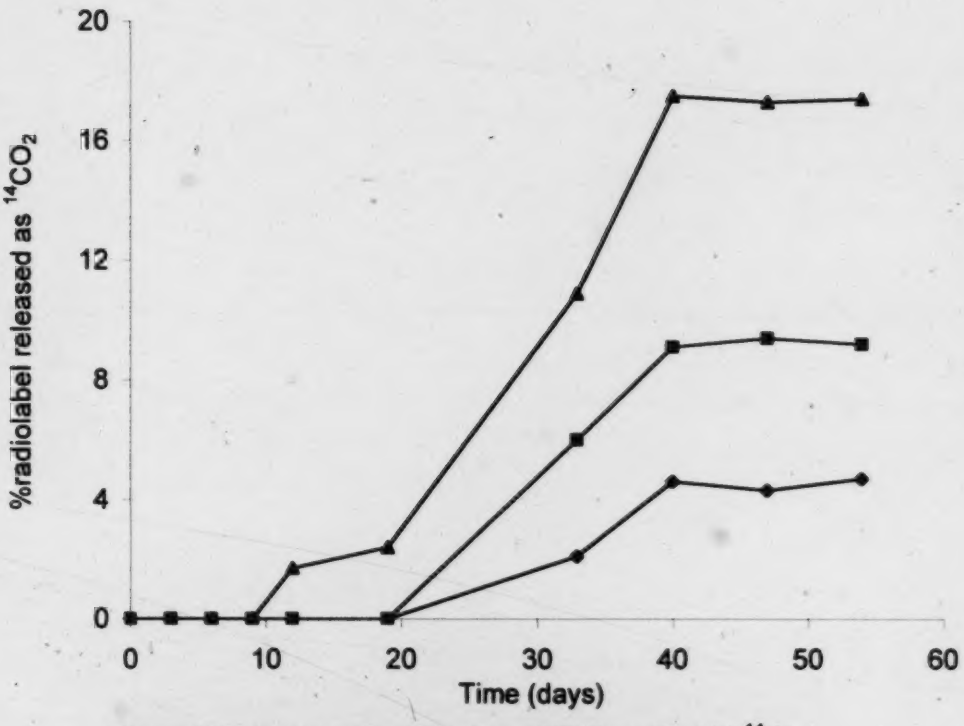


Figure 23. *In vivo* mineralization of TLC purified [9-^{14}C]-phenanthrene by *C. gallica*

Killed cells — cells in KBF — cells in KBF+ABTS+HBT

al., 1991). It was shown here that there is extensive mineralization of phenanthrene in the presence of elevated levels of laccase and in the presence of mediated laccase activity. The laccase may be responsible for the mineralization of phenanthrene because there is even more mineralization in the presence of increased laccase content and there is more mineralization when mediating substrates are added. The mediating substrates have been shown in the past to help laccase overcome substrate ionization potential barriers and so this is indirect evidence that the mediating substrates are helping laccase to overcome substrate barriers of *C. gallica* laccase. Phenanthrene is not typically considered a substrate of laccase or other ligninolytic enzymes as it has a high ionization potential (Collins et al., 1996). Laccase is an enzyme able to oxidize compounds with a very low ionization potential. It has been shown that in the presence of mediating substrates, phenanthrene and other such PAHs were substrates of laccase, as was shown here. The mineralization of phenanthrene was more significant when mediating substrates were present. This suggests that as mediating substrates increase the amount of mineralization, laccase may be involved in the degradation process. The increased mineralization in the presence of mediating substrates is not attributed to increased laccase content but to the effect of mediating substrates on laccase activity. Mediating substrates tend to increase the Kcat of the enzyme and increase the substrate range by providing an intermediate for electron transfer: it is easier to remove an electron from a mediating substrate than from the actual substrate but when the mediating substrate has been oxidized to its free radical it can remove an electron from a PAH which is not a usual substrate for laccase.

In comparing the data from the mineralization of purified phenanthrene to the other PAHs tested it is interesting to note that the incubation time for the latter is much shorter. Mineralization of the purified phenanthrene did not proceed until after day 20 whereas the other mineralization experiments were ceased as 15 days. Longer incubation times might have produced significant mineralization.

Knowing that *C. gallica* laccase can oxidize PAHs and may assist in mineralization of PAHs, the enzyme was produced in sufficient quantities for purification, characterization and further PAH metabolism studies.

4.3 Large-scale production of laccase.

In order to produce sufficient laccase for the proposed characterization and modification studies, large-scale production was investigated using optimal production conditions. Initially, *C. gallica* was grown in 2 L shake flasks with 1 L of medium to the maximum laccase activity of ~8 units/ml at 8 d. There was variability in the final laccase content between flasks of between 4 - 10 units/ml. The pH remained constant during the growth period. The sugar content decreased concomitant with the production of laccase. Only when the sugar content substantially decreased did laccase production increase significantly. A similar trend was seen when *C. gallica* was grown in a fermentor to the maximum laccase activity of 15 units/ml at 6 d, as seen in Figure 24. The pH remained constant during the growth period. When sugar content decreased there was a concomitant increase in laccase content. A 14 L fermentor with 8 L of production medium is pictured in Figure 25.

Once easily usable carbon sources such as glucose and other sugars were consumed, the fungus switched to another carbon source. The bran flakes medium provided a carbon source in the form of lignin. In order for the fungus to utilize this carbon source it secreted enzymes that are able to break down this large amorphous compound. This fungus produced laccase for this purpose. For this reason we see an increase in laccase production once sugars were exhausted. This occured in both the shake flasks and the fermentor.

Fermentor production yielded better laccase production than shake flask production. Laccase production in the fermentor is one of the highest levels of laccase production reported by a white-rot fungus. A 100 L fermentor of *P. cinnabarinus* was successfully incubated and activity reached about 18 units/ml. In comparison to shake flasks this was a two fold increase and maximum activity was reached three days earlier (Eggert *et al.*, 1996). A 5 L fermentor with 3 L of

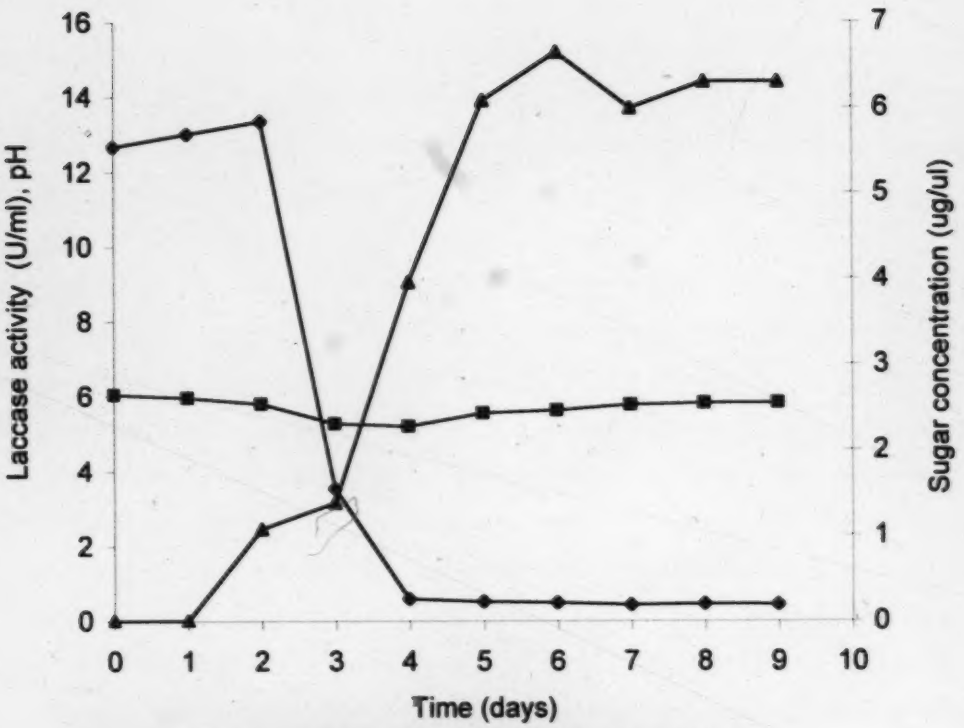


Figure 24. Large-scale production of laccase in a 14L fermentor with 10 L of 3% KBF in phosphate buffer, pH 6.5

—■— medium pH —▲— laccase activity —◆— sugar concentration

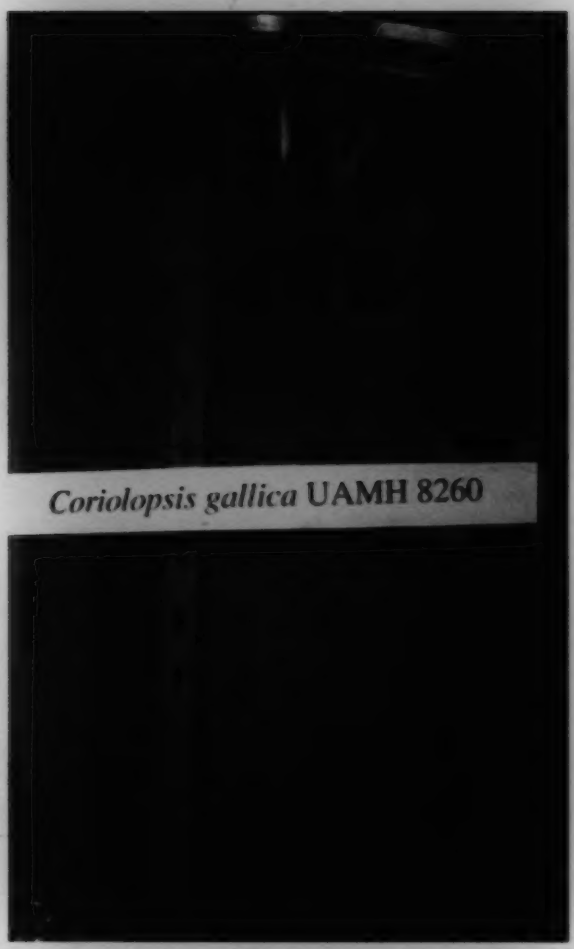


Figure 25. Photograph of a 14 L stirred tank reactor with 10 L of production medium (3% KBF in phosphate buffer, pH 6.5) with *C. gallica*.

culture medium was successfully run with *P. eryngii*. Cultivation in the fermentor increased laccase levels 2.3 fold compared with levels in 250 ml flask cultures. Laccase activity was minimal at 120 munits/ml (Munoz *et al.*, 1997). *C. gallica* A-241 IJFM was grown in medium containing paper effluent, reaching maximum values on day 5 at 6.6 units/ml (Calvo et al., 1998). A trend with scaling up production seems to be an increase in laccase production when produced in larger amounts, usually about two fold. This trend holds true for *C. gallica* laccase production where an increase of laccase production is ~2 fold greater in the fermentor.

When using fermentors, there is closer control of environmental factors such as temperature, pH and dissolved oxygen, which can result in faster growth and probably higher biomass yields, which can translate into more enzyme earlier in the fermentation process. This stands true for the comparison of shake flasks and fermentors. Laccase activity appears earlier in the fermentor. Glucose is spent earlier indicating biomass increases earlier. Due to this greater amount of biomass there is more enzyme production. There is greater variability in enzyme production in shake flasks as evident in the range of activities reported for the different shake flasks. There is one vessel for a fermentation so this variability is eliminated. Fermentors are less labor intensive and produce higher enzyme activity in a shorter period of time. From an economic viewpoint, the fermentor has many advantages over shake flask production. It seems clear that the best production of enzyme is in a stirred tank reactor with production medium.

Some parameters that should have been followed along with laccase activity, pH and sugar content are protein content and biomass. Protein measurements were not obtained as Kellogg's bran flakes' medium contains a high content of proteins at 10% w/w content. Levels of enzyme production would be orders of magnitude less than the proteins already present in the medium. Any measurement employed would not be sensitive enough to detect minimal increases. Biomass was also difficult to measure as the bran flakes in the

medium were not always completely digested. It was difficult to separate bran flakes from the biomass pellets to obtain accurate biomass measurements. These values would have provided valuable information into the production of the enzyme.

4.4 Laccase purification.

Once significant laccase had been produced, more than 100,000 units, a purification was attempted based on published schemes. A purified laccase from *T. versicolor* was used for comparison. It was part of a laccase preparation used by Pickard and Westlake (1970) and had been frozen for 30 y. It was known that the preparation contained two major isoenzymes named laccase A and B after their elution positions. The laccase from *C. gallica* was purified according to the procedure summarized in Table 5. The concentrated broth from the bran flakes medium was contaminated by pigments, which were largely removed by anion-exchange (DE52) column chromatography, as in Figure 26. The laccase fraction eluted at 25 mM NaCl. To remove further pigments the concentrate was passed through a second anion exchange (DE52) column, as in Figure 27. The laccase fraction eluted at 25 mM NaCl. As the enzyme solution appeared green it was passed through a G100 gel filtration column, as in Figure 28. The laccase fraction was blue. Overall, the enzyme was purified 18.5-fold with a 77% recovery of the laccase based on the Bradford protein assay. Purification of laccase to this point was followed by SDS-PAGE as seen in Figure 29. There are fewer bands in each of the lanes as the laccase is subjected to purification. The disappearance of bands is most noticeable in the last column step, gel filtration. It is clear that the procedure utilized is removing contaminating proteins from the laccase. The pigment was heterogeneous in molecular size as shown by gel filtration and charge distribution as shown by ion exchange chromatography. For this reason additional purification steps were introduced, depending on the amount of pigment in the culture supernatant.

Pigments including an orange pigment were produced by the fungus during growth on bran flakes giving the medium a dark brown color. These are

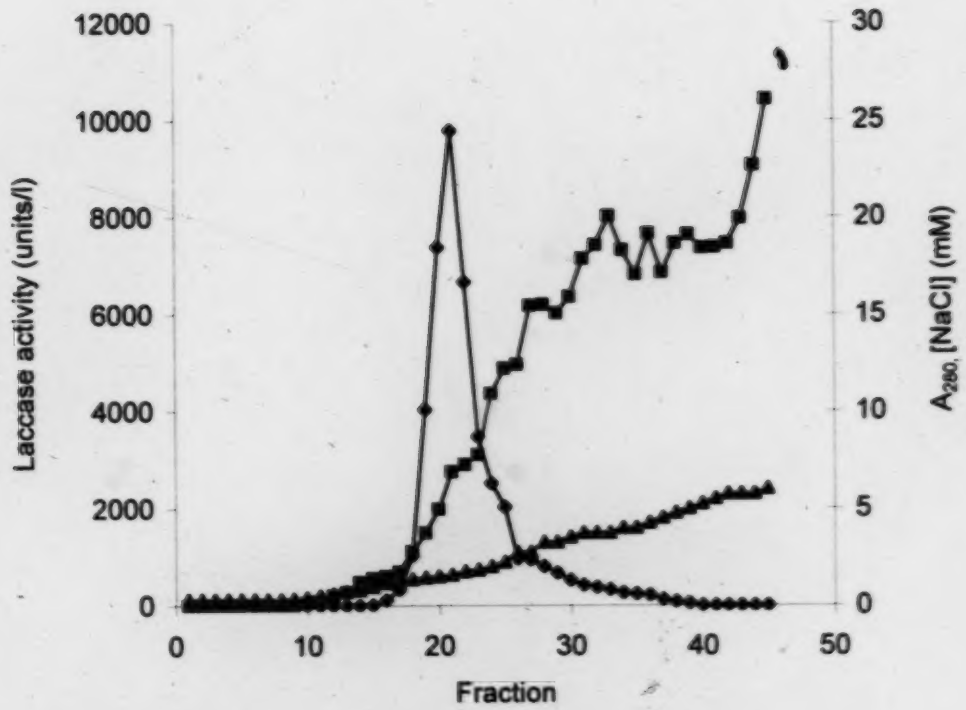


Figure 26. Elution profile of laccase from a 500 ml DE-52 anion exchange —◆— Enzyme Activity —■— A280 —▲— [NaCl] (10x)

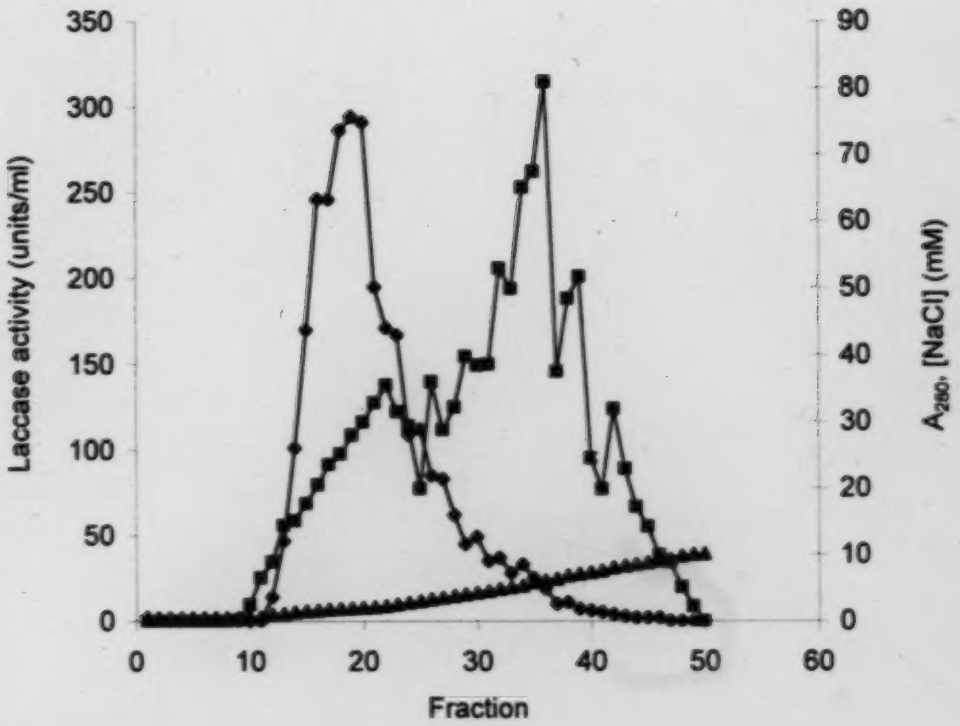


Figure 27. Elution profile of laccase from a 200 ml DE-52 anion exchange column —◆— Laccase Activity —▲— [NaCl] (10x) —■— A280

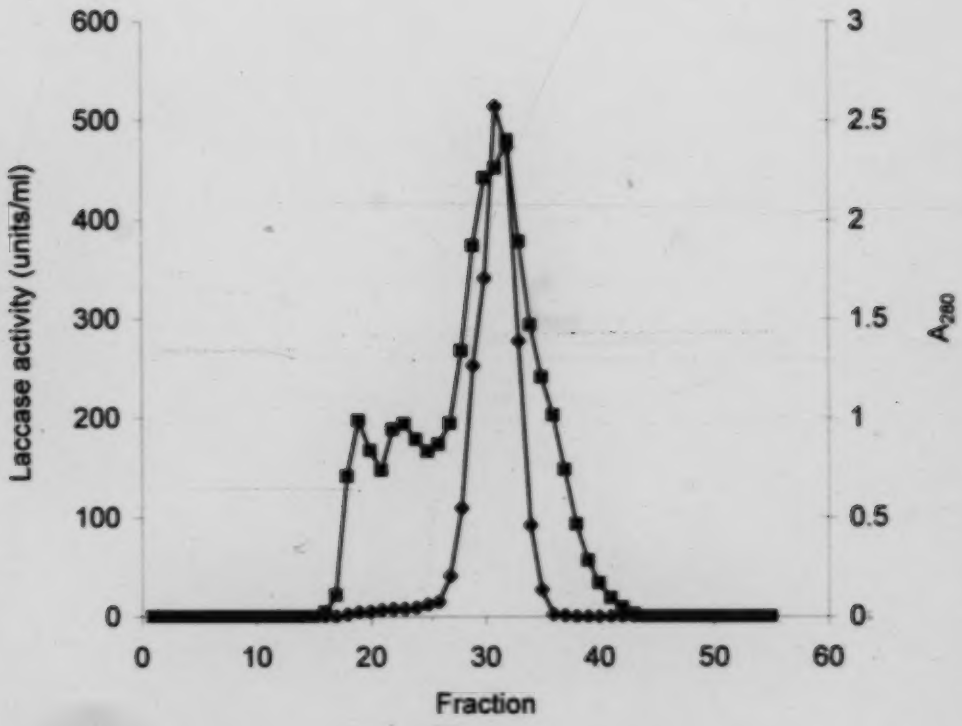


Figure 28. Elution profile of laccase from a 100x2.5cm G-100 gel filtration column —◆— Enzyme Activity —■— A280

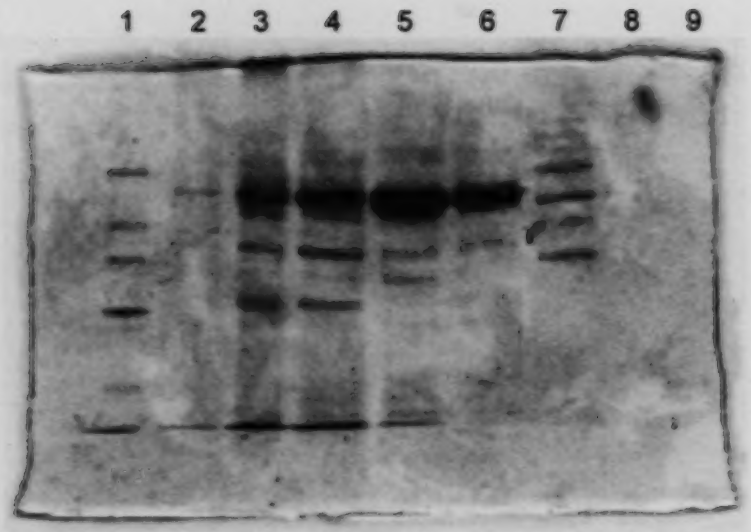


Figure 29. SDS-PAGE gel of purification procedure of laccase from *C. gallica*.

Lane 1: low molecular weight markers (5 μg protein)

Phosphorylase B	97,400 Da
Bovine serum albumin	66,200 Da
Ovalbumin	45,000 Da
Carbonic anhydrase	31,000 Da
Trypsin inhibitor	21,500 Da
Lysozyme	14,400 Da

Lane 2: culture medium supernatant (10 μg protein)

Lane 3: culture mediun concentrate (10 μg protein)

Lane 4: DE52 pool 1 (5 μg protein)

Lane 5: DE 52 pool 2 (5 μg protein)

Lane 6: G100 gel filtration pool (5 μg protein)

Lane 7: High molecular weight markers (5 μg protein)

Myosin	200,000 Da
B-galactosidase	116,250 Da
Phosphorylase	97,400 Da
Bovine serum albumin	66,200 Da
Ovalbumin	45,000 Da

the major contaminants of the supernatant solution as seen by the SDS-PAGE gel. There are several bands in the concentrate and even after passage through DE52 columns the contaminating bands are still present to a similar degree. The first steps are meant to eliminate pigments. These pigments along with protein have an absorbance at 280 nm.

The initial FPLC strong anion exchange column utilized was a High Q column (Pharmacia). The High Q column was first used with *T. versicolor* laccase to determine the best resolving and recovery conditions for isoenzymes. The laccase of *T. versicolor* was resolved into two laccase activity peaks eluting from the column which corresponded to two distinct isoenzymes, laccase A and B respectively. There was 2% recovery for laccase A and 49% recovery for laccase B. The application of *C. gallica* laccase to the High Q column produced different results. Less than 0.1% applied to the column was recovered, eluting at the beginning of the run. The rest of the enzyme eluted later corresponding to laccase B, yielding a recovery of 24%.

Subsequent FPLC strong anion exchange chromatography was performed using the Mono Q column. The *T. versicolor* laccase was applied to this column first to determine the best conditions for resolving peaks and recovery of isoenzymes. The *T. versicolor* laccase eluted in two activity peaks as seen in Figure 30. The first peak (fractions 2-5) contained 2.9% of the initial enzyme activity and the second peak (fractions 36 –50) contained 71.6% of the initial activity applied to the Mono Q column. The recovery was similar to the High Q column. However when *C. gallica* was applied to the Mono Q column there was an increase in the enzyme recovery, 92% of the initial enzyme activity eluted from the column, in one activity peak (fractions 24-36) compared to the High Q column. There was no activity eluted at the beginning of the salt gradient as seen in Figure 31. The fractions from the Mono Q were bright blue after concentration. It appears that the Mono Q column is superior for recovery of *C. gallica* laccase.

The progress of the laccase purification can be seen by SDS-PAGE. The

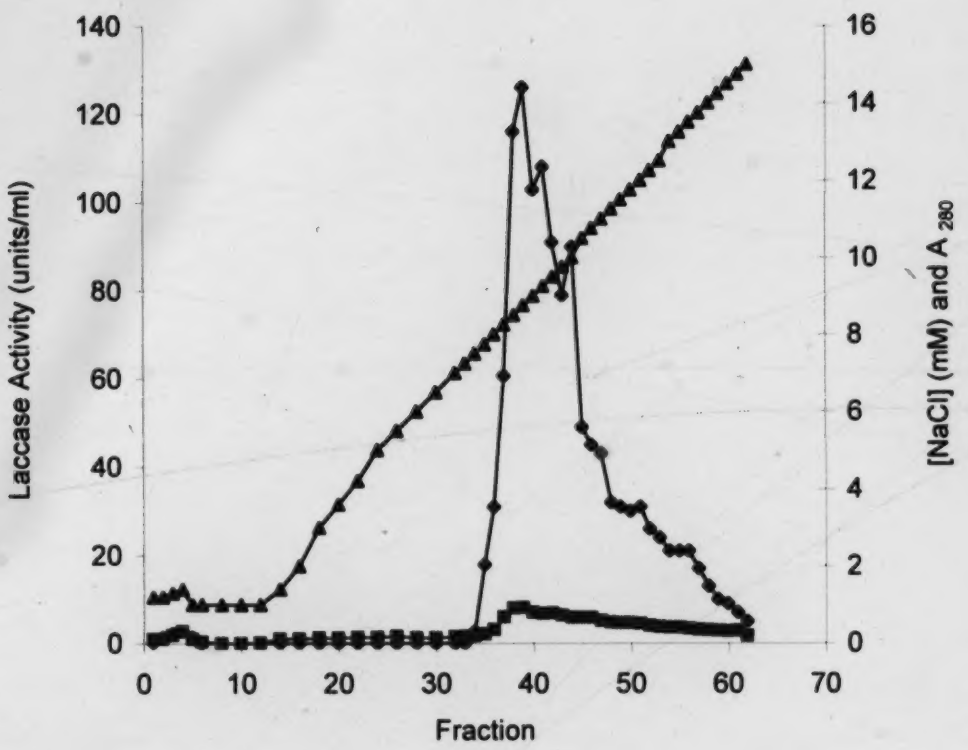


Figure 30. Elution profile of *T. versicolor* laccase from a Mono Q column —◆— Laccase activity —■— A 280 —▲— [NaCl]

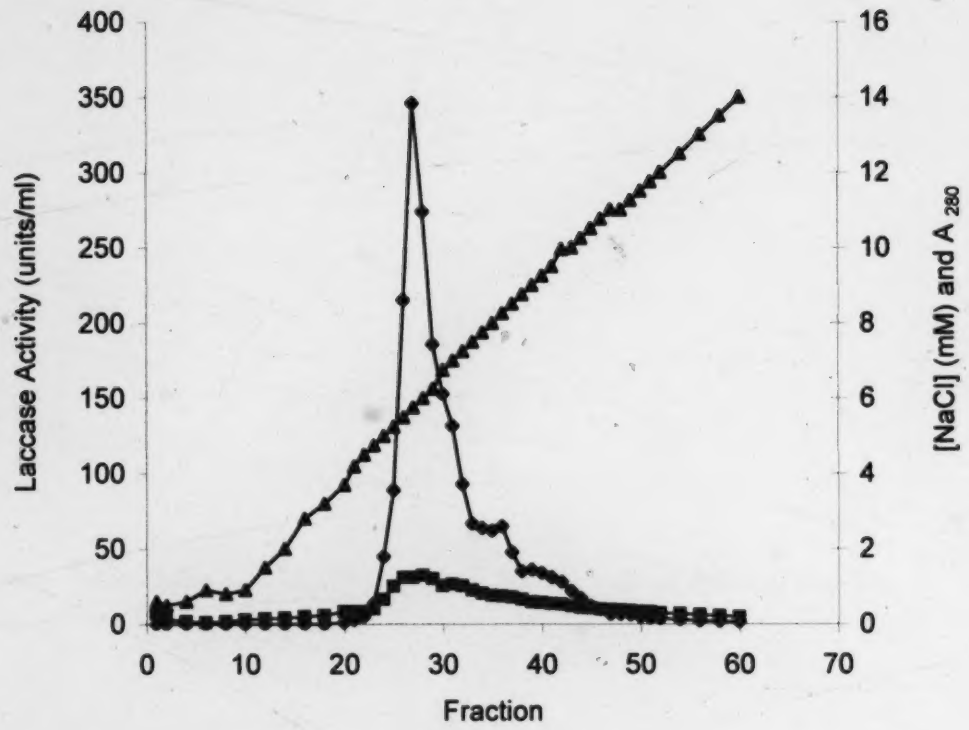


Figure 31. Elution profile of *C. gallica* laccase from a Mono Q column —◆— Laccase activity —■— A 280 —▲— [NaCl]

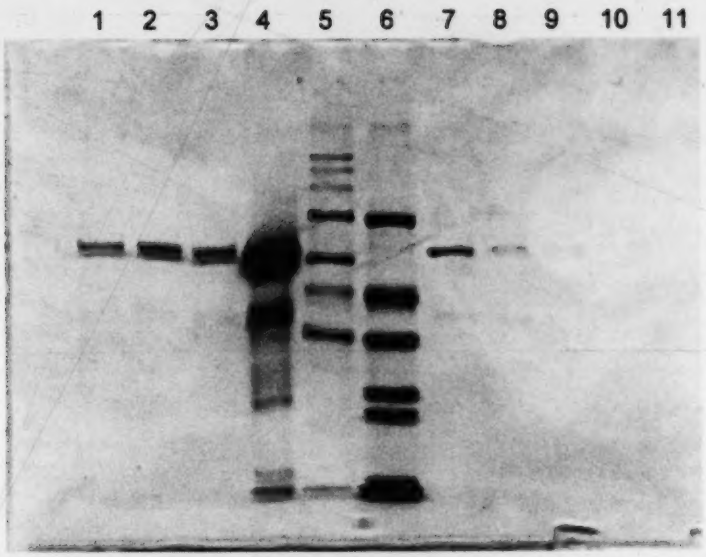


Figure 32. SDS-PAGE gel of Mono Q purification of *C. gallica* laccase.

Lane 1: Fraction 24 (5 μg protein)

Lane 2: Fraction 26 (5 μg protein)

Lane 3: Fraction 28 (5 μg protein)

Lane 4: DE52 pool 2 (5 μg protein)

Lane 5: High molecular weight markers (5 μg protein)

Myosin	200,000 Da
B-galactosidase	116,250 Da
Phosphorylase	97,400 Da
Bovine serum albumin	66,200 Da
Ovalbumin	45,000 Da

Lane 6: Low molecular weight markers (5 μg protein)

Phosphorylase B	97,400 Da
Bovine serum albumin	66,200 Da
Ovalbumin	45,000 Da
Carbonic anhydrase	31,000 Da
Trypsin inhibitor	21,500 Da
Lysozyme	14,400 Da

Lane 7: Fraction 30 (5 μg protein)

Lane 8: Fraction 32 (5 μg protein)

purification was monitored after passage through the Mono Q column. The pure laccase produced one major band on an SDS-PAGE gel as seen in Figure 32. Several key fractions were run on the gel along with the laccase preparation. The laccase preparation ran on the SDS PAGE gel as one strong band with a few minor background contaminants as seen in Figure 32. The purification of the enzyme was repeated until there was sufficient purified enzyme for further studies.

The yield of the enzyme overall was 75.4%. This yield is as good or better than yields for other purification procedures. Some procedures have yielded recoveries of between 25-35% after Mono Q column chromatography whereas upwards of 92% recovery was obtained from this purification.

The yield from the other columns is comparable to other purification of laccase. Recovery of *P. ostreatus* laccase from Sephadex G-100 column is in a range of 45-90% (Palmieri et al., 1997). Yields from anion exchange columns have a range of 54-79%. For another strain of *C. gallica* the recovery was very low after DEAE-Biogel at 8%, and after Mono Q at 4.1% (Calvo et al., 1998). The yields achieved here are much higher.

The purification factor for this enzyme is about 18.5 fold. This is lower than other factors at 43. This variable indicates the amount of purification of the target protein from other proteins. The main contaminants of this laccase preparation were pigments produced by the fungus during growth and not other proteins. This is reflected in the lower purification factor as the pigments are likely not measured by the protein measurements using the Bradford method. The first steps in the column procedure were anion exchange (DE52) and this removed many of the contaminating orange pigments and some proteins. There was about a 13 fold increase in specific activity over the two anion exchange steps. According to the SDS gel there is limited purification of other proteins within the first two steps. The gel filtration step removes any proteins or compounds that are closely related. This step provides additional purification as seen on the SDS-PAGE gel where contaminating proteins are not as apparent.

The final step in purification was a strong anion exchanger such as High Q or Mono Q. Using a strong anion exchange column has proven in the past to separate enzymes with small differences in charge, as little as one charge unit difference. Any closely related ionic species which were not separated from the laccase by DE52 could be separated by this type of chromatography. This was indicated by the color of the enzyme. It has a strong blue color with a slight green color, possibly due to closely related orange pigments. When using the Mono Q column, there was no increase in specific activity in this step and a small increase in purification fold, indicating that proteins were not purified as much as pigments were removed from the enzyme fraction.

For *T. versicolor*, it has been shown that there are two isoenzymes that can be separated by chromatographic methods. For the *T. versicolor* laccase, the experimental protocol separated the isoenzymes. When the procedure was done on *C. gallica* laccase, one activity peak was resolved corresponding to one isoenzyme. It ran in a position similar to laccase B of *T. versicolor*. The enzyme was judged ready for characterization and use for other procedures when the specific activity remained constant and there was one major band on SDS-PAGE with a few minor bands.

Native gels were utilized to look at activity staining and purification. Two well known substrates of laccase were used, ABTS and *o*-dianisidine. Each of the stains gave very quick colored reactions on the gels, green and brown respectively, as seen in Figure 33. ABTS stained gels gave a very diffuse result. The color was very intense but the stain diffused through the entire gel making it difficult to read the results.

The *o*-dianisidine stained side was not as diffuse and gave very intense colors. The gel could be read and discrete bands could be seen. In lane one there was a long smear with areas that were more intense. As this mixture should contain the isoenzymes of laccase for *T. versicolor* it seemed that separation of isoenzyme on this gel was possible. The second lane showed one area of staining. The third lane showed another area of staining in a different

Table 5. Purification of laccase from C. *gallica*.

Step	Volume (ml)	[Protein] (mg/ml)	Activity (U/ml)	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	4700	0.062	14.2	291	66740	229	1	100
Ultra-filtration	384	0.635	168.3	244	64608	265	1.2	96.8
DE52-1	8.5	4.5	7543	38.3	64531	1685	7.4	96.7
DE52-2	4.8	4.46	13413	21.4	64382	3009	13.1	96.4
Gel Filtration	6.2	2.02	8259	12.1	51206	4232	18.5	76.7
Mono Q	6.5	1.70	7740	11.1	50310	4532	19.8	75.4

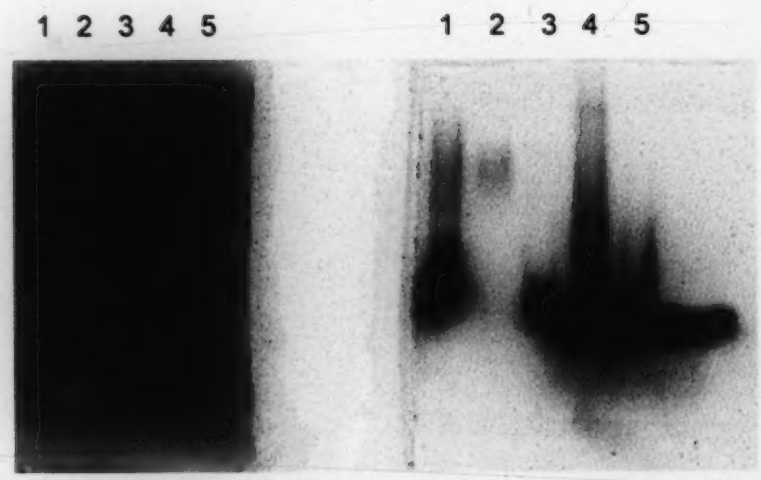


Figure 33. Activity staining of native PAGE gel with ABTS (left) and *o*-dianisidine (right).

Lane 1: *T. versicolor* pre-mono Q (5 μg protein)

Lane 2: *T. versicolor* laccase A (5 μg protein)

Lane 3: *T. versicolor* laccase B (5 μg protein)

Lane 4: *C. gallica* pre-mono Q (5 μg protein)

Lane 5: *C. gallica* laccase B (5 μg protein)

position. These corresponded to the two isoenzymes of *T. versicolor*. The application of *C. gallica* showed in all lanes that there was one area of staining. Further attempts to reduce the amount of enzyme applied did not reduce the amount of leaching of the color. The solubility of *o*-dianisidine upon reaction with laccase is much lower than the solubility of ABTS , and therefore the stain will not diffuse as much and keep to the bands of protein. After reaction with laccase, *o*-dianisidine polymerizes to produce an insoluble product so it will not leach out of the gel as rapidly as the product of ABTS, which does not polymerize. It was clear from the data that laccase was present in a homogeneous solution and was ready for characterization.

4.5 Characterization of laccase.

4.5.1 Molecular weight.

In order to understand the processes of PAH degradation an understanding of the enzyme is necessary therefore physical and chemical characteristics of the enzyme were investigated. Using SDS-PAGE an estimate of the molecular weight of laccase was determined. Figure 32 shows an SDS-PAGE gel with molecular weight markers. According to this gel the laccase is a 66 kDa protein. However migration of glycoproteins on an SDS-PAGE gel can be affected by its sugar moieties, so this is probably not an accurate value.

A second method used to determine molecular weight was MALDI-TOF mass spectroscopy. The molecular weight of *C. gallica* laccase was 56688 Da and *T. versicolor* laccase was 60151 Da as seen in Figure 34 A and B respectively. This technique is not affected by the carbohydrate content of a protein and for this reason is considered to be more accurate. Other values for laccase determined by MALDI-TOF mass spectroscopy are for *P. ostreatus* ATCC 58053 at 55.5 KDa and *P. ostreatus* UAMH 7988 at 55.35 KDa (P. Reimer, unpublished). As well a laccase from *P. ostreatus* (strain Florida) was analyzed by both SDS-PAGE and MALDI-TOF mass spectroscopy. The two isoenzymes were determined to be 61 KDa and 67 KDa by SDS-PAGE and the second isoenzyme was determined to have a broad peak centered around 61373

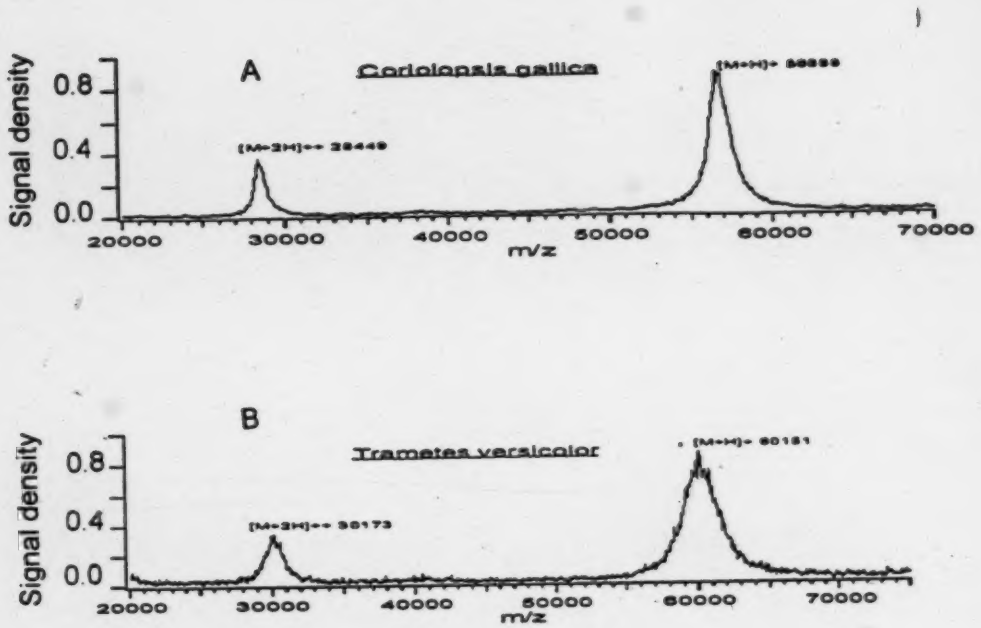


Figure 34. MALDI-TOF mass spectra of laccases isolated from (A) *C. gallica* and (B) *T. versicolor*. The $[M+2H]^{++}$ peak indicates half the molecular weight, as the protein has been doubly ionized. The $[M+H]^{+}$ peak indicates the molecular weight of the protein, as the protein has been singly ionized.

Da by MALDI-TOF mass spectroscopy (Palmieri et al., 1997). The value determined by MALDI-TOF mass spectroscopy was smaller than the value determined by SDS-PAGE: this is the same trend that was observed for *C. gallica* laccase in this study.

The reported molecular weight of laccase ranges from 50 to 80 kDa. Several laccases have been analyzed for molecular weight by SDS-PAGE including *P. cinnabarus* (76.5 KDa), *P. eryngii* (65 and 61 KDa), *Panaeolus sphinctrinus* (60 KDa), *C. gallica* A-241 (84 KDa), *Marasmius querocophilus* (63 KDa) (Calvo et al., 1998; Dedeyan et al., 2000; Eggert et al.. 1996; Farnet et al., 2000; Munoz et al., 1997, Heinzkill et al., 1998). This is not a comprehensive list but gives an idea of the range of molecular weights determined for laccases. The values from both techniques are well within the ranges of previously characterized laccases. The SDS-PAGE value is larger than the MALDI-TOF value. This is probably due to the carbohydrate content of laccase. Binding of SDS to the carbohydrate portion of the protein is unpredictable. It is the uniform binding of SDS to the polypeptide that makes the charge to mass ratio consistent for all proteins so that the property of the protein being examined is the molecular weight. Since SDS binding to the carbohydrate portion is variable this charge to mass ratio may be affected and other factors are associated with migration in the gel thereby giving anomalous measurements.

4.5.2 Copper content.

The blue color of laccase is due to its Cu content. Cu atoms are an important cofactor and require consideration when studying the features of laccase. The laccases of ligninolytic fungi generally contain four Cu atoms, one type 1, one type 2 and two type 3. This laccase seems to be typical. Spectral analysis of purified *C. gallica* laccase showed that there is a shoulder at 330 nm corresponding to a type 3 binuclear Cu, a peak at 606 nm, corresponding to a type 1 blue Cu atom and a protein peak at 280 nm as seen in Figure 35. The laccase of *P. cinnabarinus* had similar spectroscopic data along with *Rhus vernificera*, *Podospora anserina* and *P. eryngii* (Eggert et al., 1996, Munoz et al.,

1997). The light absorption spectrum of *C. gallica* laccase is a typical laccase spectrum suggesting the presence of typical Cu atoms.

The amount of Cu was 3.5 atoms per molecule as determined by the 2,2'-biquinoline assay using the molecular weight of 56 KDa. This is a reasonable estimation for laccase as all laccases characterized have been shown to have one type 1 Cu, one type 2 Cu and two type 3 Cus. One Cu atom may be bound too tightly to the protein to be detected by the assay method or lost during purification. For this reason, the value of Cu obtained was slightly lower than expected. Laccase of *C. subvermispora* was determined to contain 3.7 mol of Cu/mol of protein (Fukushima and Kirk, 1995). Laccase molecules of *Coriolus zonatus* was determined to contain 3.5–3.9 Cu atoms (Koroljova et al., 1999).

The 280 nm/600 nm absorbance ratio was 10. The ratio of A_{280} to A_{600} is indicative of the purity of the enzyme preparation. This ratio reflects the balance between the absorbance of a type 1 Cu atom at 610 nm and the combined absorbance of tryptophan and tyrosine residues of the protein at 280 nm. As the value gets smaller there is more Cu for protein so the smaller the number, the purer the enzyme preparation. Other values in the literature have shown values of 17.0 to 132 but the typical blue laccase range is between 15-20. *C. subvermispora* laccase preparation had a ratio of 20 (Fukishima and Kirk, 1995), for *P. eryngii* laccase preparation, the ratio was 15 (Munoz et al., 1995) and for *T. versicolor* laccase preparation, the ratio was 18 (Bourbonnais et al., 1995). The value of 10 for this enzyme preparation is much lower than other values, therefore it is either deemed to be purer than other preparations, or *C. gallica* laccase has lower aromatic content (tryptophan and tyrosine) than other laccases.

4.5.3 Carbohydrate content.

All laccases characterized to date are glycoproteins and this characteristic imparts resilient properties to laccases. *C. gallica* laccase has 21% carbohydrate

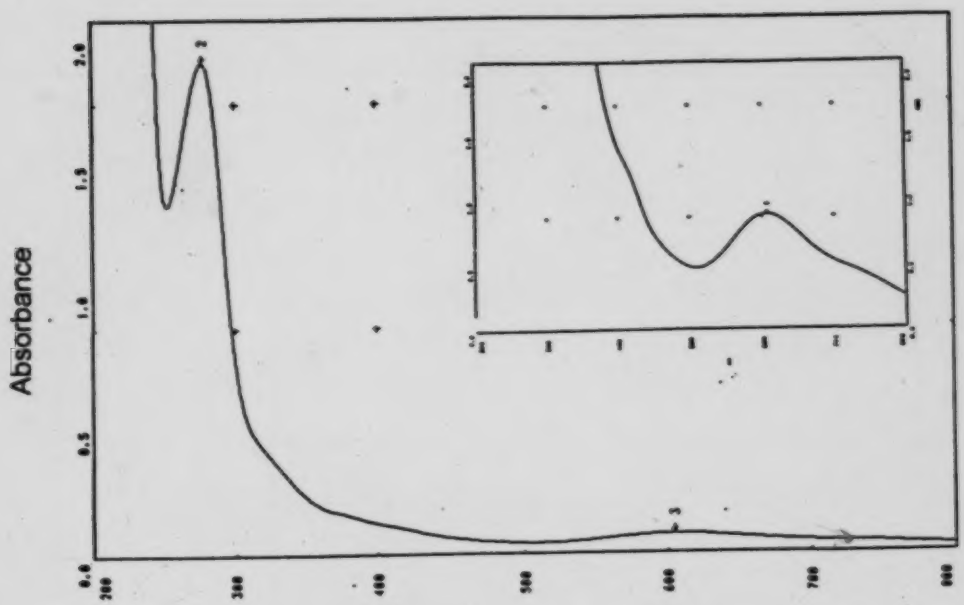


Figure 35. UV-visible spectrum of *C. gallica* laccase. The two peaks at 280 nm and 600 nm correspond to the protein peak and the blue type 1copper peak. A shoulder at 320 nm corresponds to the type 3 copper atom peak. Inset, the blue copper peak increased in size.

as compared to *T. versicolor* laccase which has 14% carbohydrate when assayed in this laboratory. The growth conditions of the fungus can have a marked effect on the amount of glycosylation, as seen in a study of chloroperoxidase (Pickard and Hashimoto, 1988). These values may not be directly comparable due to the fact that the fungi producing the enzymes were grown under different conditions.

The estimated carbohydrate contents of laccase are slightly higher than the range reported for the laccase of other basidiomycetes, including *T. versicolor*, *Coriolus hirsutus*, *P. radiata*, *Agaricus bisporus* and *Armillaria mellea* (Fukishima and Kirk, 1995). *P. eryngii* has two isoenzymes with carbohydrate content of 1% and 7% (Munoz et al., 1997). *C. subvermispora* laccases had an approximate sugar content of 15% and 10% for L1 and L2 isoenzymes respectively (Fukishima and Kirk et al., 1995). *C. zonatus* laccase had 10% carbohydrate per mole of protein (Koroljova et al., 1999).

Relative glycosylation levels can impart different properties to enzymes. It has been demonstrated that the carbohydrate moiety of the laccase of *T. versicolor* imparts resistance to proteolytic attack and elevated temperatures (Yoshitake et al., 1993). The resistance to proteolytic attack can be attributed to the specificity of proteases as it will specifically cleave the peptide bonds in the primary structure but the carbohydrate can obscure the peptide bonds, thereby conferring resistance. The carbohydrate can also confer thermophilic stability by additional hydrogen bonding between the carbohydrate portion and the protein. Perhaps the somewhat greater thermal stability of *C. gallica* laccase over other laccases reflects its higher than average carbohydrate content.

4.5.4 N-terminal amino acid sequence.

The N-terminal amino acid sequence for *C. gallica* was determined and was homologous to other laccase sequences as seen in Table 6. There were a few unique residues. The closest relative to *C. gallica* laccase was another strain of *C. gallica* with a single amino acid difference but with several amino acids missing. *C. hirsutus* displayed 3 amino acids that were different among the 20

laccases compared. *Coprinus friesii* was also close with six differing amino acids. Similar to other laccases, *C. gallica* laccase N-terminal amino acid sequence displayed homology to other sequences of WRF laccases. In contrast, the N-terminal sequences of laccases isolated from non-ligninolytic fungi such as the commercial mushroom, *A. bisporus*, the ascomycete, *Neurospora crassa*, and the yeast, *Cryptococcus neoformans* were significantly different.

The N-terminal amino acid sequence was determined in order to acquire information about the primary structure of the proteins. Comparison of this sequence to other laccases indicated that it is homologous to other laccases. The N-terminal sequence typically is part of the Cu binding structure. As mentioned earlier, the mode of action of laccases through the copper domains is similar, and the N-terminal amino acid sequence contains histidines which form part of the copper-binding structure. Since the first 20 amino acids of the N-terminal sequence are similar it may be extrapolated that the Cu-binding amino acid residues are similar.

4.5.5 Isoenzymes and isoelectric point.

There is strong evidence that the laccase from *C. gallica* is produced as a single acidic isoenzyme, as only one distinct protein peak was observed with all protein separation and analytical techniques. From the purification procedure the Mono Q column eluted one peak of laccase activity. This column has been shown to resolve isoenzymes. The Mono Q successfully separated the two isoenzymes of *T. versicolor* therefore indicating that *C. gallica* produced one isoenzyme. The native gel also indicated that there was one isoenzyme, as one band was visualized. The native gel displayed two regions of staining for *T. versicolor*. Finally, isoelectric focusing of the *C. gallica* enzyme presented one isoenzyme, as indicated by one band on an isoelectric focusing gel. The isoelectric point (pI) of this isoenzyme was difficult to determine as the pI was at the low end of the acidic range. The only conclusion that can be drawn is that it is acidic and that the isoelectric point is below pH 3.0 as this is

Table 6. N-terminal amino acid sequence of fungal laccases.

Microorganism	N-terminal amino acid sequence
C. gallica	A I G P V A D L T I S N G A V S P D G F
C. gallica A-241	S I G P V A - L T I S N G - V - P
Coriolis hirsutus	A I G P T A D L T I S N A E V S P D G F
Coprinus friesii	A I G P V A D L Y I G N K V I A P D G F
Pleurotus ostreatus	A I G P A G N M Y I V N E D V S P D G F
Ceriporiopsis subvermispora	A I G P V T D I E I T D A F V S P D G P
Coriolus. versicolor	G I G P V A D L T I N A A V S P D G F
P. radiata	S I G P V T D F H I V N A A V S P D G F
P. cinnabarinus	A I G P V A D L T L T N A A V S P D G F S
P. tremellosa	A I G P V T N F H I V N A I A A P D G F
Panus tigrinus	A V G P V A D L T V T N A N I S P D G F E
Panaeolus sphinctrinus	A I G P V A D L Y I G N K V I A P D G F
Panaeolus papilionaceus	G I G P V A D L T I T N A A V S P D G F
Polyporus pinsitis	G I G P V A D L T I T N A A V S P D G F
Agaricus bisporus	A K T R T F D F D L V N T R L A P D G F
N. crassa	G G G G G C N S P T N R O C W S P
Cryptococcus neoformans	X K T D E S P E A V S D N Y M P K
Chaetomium thermophilium	F N P D L L P S L E P

the lower limit of the markers and the enzyme was well below these markers. Having only one acidic isoform is an unusual feature among fungal laccases. *P. cinnabarinus* was one other fungus found to have one isoform (Eggert et al., 1996), but most other fungal laccases were found to have numerous isoenzymes. *P. ostreatus* laccase was passed through a DEAE sepharose column and was separated into three distinct protein fractions (Palmieri et al., 1993). Since the pH during the degradation of lignin in wood gradually decreases from 5 to 3.5, the different pH optima of isoenzymes allow oxidation over a wider pH range. Multiple isoenzymes that are produced tend to display differences in stability and catalytic features. The larger pH range of this enzyme allows for a similar range to be covered with the use of one isoenzyme. The extended stability and catalytic features of the single *C. gallica* laccase enzyme can encompass the same features as multiple isoenzymes.

This is different from results previously determined for *C. gallica*. A second isoenzyme was separated using High Q anion exchanger (Rodriguez et al., 1999). There was some laccase activity that detected at the elution front of the High Q column, but this was less than 0.1% and was probably follow-through from overloading the column with protein and other ionic species, such as pigments. This is probably not a second isoenzyme. If it is, it is a minor form under these growth conditions and would not significantly contribute to activity and function of laccase.

In other bran based media, two isoenzymes were produced, whereas in minimal medium there were multiple isoforms detected (Fukishima and Kirk, 1995). It has been speculated that many isoforms are produced to deal with limitations of other isoenzymes. Differences in characteristics of isoenzymes such as pH optimum and substrate specificity are useful for dealing with changes in medium and being able to use a heterogeneous medium. The lack of multiple isoenzymes in *C. gallica* is not a surprise as the bran flakes medium contains one major source of carbon (lignin) and therefore would require only one isoenzyme.

4.5.6 Studies on pH.

The enzyme was tested for its characteristics under different pH conditions. The optimum pH of activity for *C. gallica* laccase was pH 4.0 for the oxidation of ABTS as seen in Figure 36. The optimum pH for *T. versicolor* laccase was 3.8. Laccase activity was lower at other pHs as pH either increased or decreased.

Fungal laccases are generally active at low pH values, in the range of 3 to 5 (Chefetz et al., 1998). One fungus, *C. thermophilium*, has been shown to have an optimum at pH 6-8 for the oxidation of ABTS (Chefetz et al., 1998). *T. versicolor* pH optimum has an experimental value of 3.8. This is well within the range of other fungal blue laccases. *C. subvermispora* laccases L1 and L2 had pH optima of 2 to 3 for the oxidation of ABTS (Fukushima and Kirk, 1995). *P. cinnabarus* was shown to have a optimum pH of 4 for ABTS (Eggert et al., 1996). *P. ergynii* had a pH optimum of 4.5 (Munoz et al., 1997). *C. gallica* A-241 had an optimal pH of 3 (Calvo et al., 1998). Many other laccases have been characterized and have been shown to have pH optima similar to *C. gallica* laccase.

The pH optimum for activity depends on the substrate chosen. For example, *P. ostreatus* has a pH optimum range of 3-3.5 for the oxidation of ABTS, but when the pH optimum is determined for guaiacol and syringaldazine it is 5.6 and 6.7 respectively (Palmieri et al., 1993). This can be attributed to the reaction mechanism of the laccase, whether the mechanism involves the abstraction of an electron or of a hydrogen. The latter will be affected by changes in pH. As well, the reaction depends on the redox potential of the substrate. The pH affects the ionization state of the substrate and therefore affects its ability to act as a reducing substrate. The test of pH optimum with ABTS gives a good indication of the characteristics of the laccase, as the steps in the oxidation of ABTS are not pH dependent within the range of pH 2-11 (Majcherczyk et al., 1999). Different substrates are affected differently by

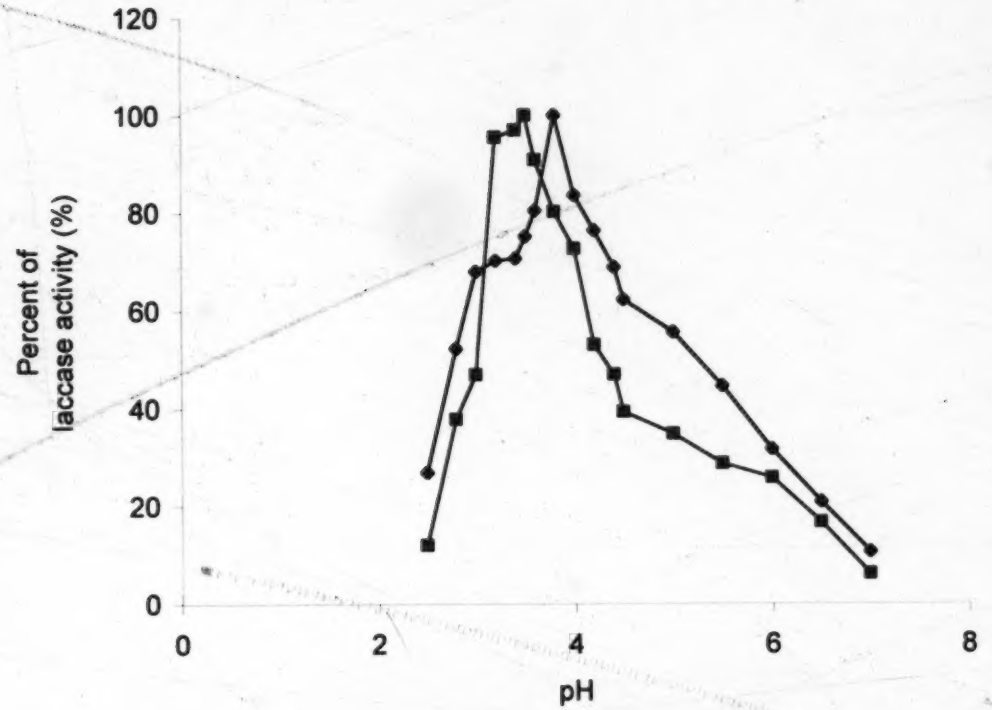


Figure 36. Studies of the effect of pH on laccase activity using purified enzymes. —◆— *C. gallica* —■— *T. versicolor*

changes in pH. So these values are viewed with caution. The pH optimum for *C. gallica* laccase cannot be extrapolated from ABTS to other substrates: for each substrate must be tested individually.

The stability of *C. gallica* laccase was also studied. The laccase was stable over a wide pH range. From pH 5.5 to pH 10 there was little appreciable loss of activity over a 5 day period. There was rapid loss of activity below pH 4.0 and at pH 3.0 loss of activity was immediate. The pH best suited for stability was pH 6.0 as there was little loss in activity over the 5 day test period as seen in Figure 37.

The wide range of pH stability of *C. gallica* laccase is higher than average for typical laccases. Typically laccases are stable from pH 4 to 7: *P. ergynii* laccase was stable from pH 3 to 10 (Munoz et al., 1997). *C. gallica* A-241 laccase was most stable at pH 4.5 which is much different from the *C. gallica* laccase in this study (Calvo et al., 1998). *P. flavido-alba* laccase was unstable above pH 3.0 (Perez et al., 1996). *P. ostreatus* laccase activity remained almost unaltered in the range of pH 5.0 to 7.0, whereas it was unstable at other pHs (Palmieri et al., 1993). The wide pH range of stability increases the suitability of *C. gallica* laccase for possible chemical modifications. These types of modification require a high pH as they tend to promote deionization of the active group involved, an amino group, which is a nucleophile only when it is deprotonated.

The amino acid composition for *C. gallica* laccase is not known but the amino acid sequence of laccases tend to be conserved particularly in regions that bind to the functional Cu atoms in the active site. The effect of pH on enzymes is due to changes in the state of ionization of the components of the system as the pH changes. Activity is affected if the catalytic group's ionization is affected by pH. This is the effect being determined by the pH optimum. The best combination of catalytic groups in terms of ionization is at pH 4.0. The bell shaped curve of the graph for pH optimum indicates that at least two ionizable groups are involved in active site catalysis. The activity can also be affected by

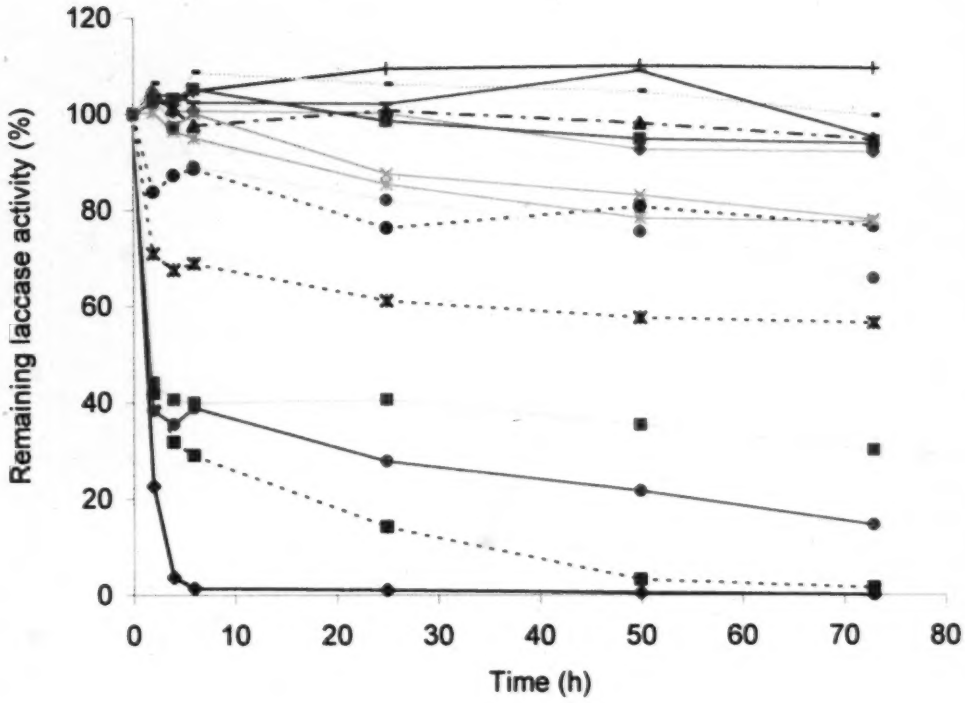


Figure 37. The pH stability of purified laccase from *C. gallica*

denaturation of the enzyme due to pH. This is the effect being studied by the stability of the enzyme in different pH conditions. Since enzymes are proteins containing many ionizable groups, they exist in a whole series of different states of ionization and the distribution of the enzymes among the various ionic forms depends on the pH and the ionization constants of the various groups. It is impossible to know the effects of amino acid residues on the stability of the enzyme by comparison with other laccases, as the complete sequence of *C. gallica* laccase is not known. Although the residues at the active site should be conserved, other amino acids will participate in stabilizing the enzyme structure in its environment. It is this variation in amino acid composition that may be the cause of the enhanced stability of this enzyme in an alkaline environment. Amino acid composition affects the secondary structure of the protein by bonding between amino acids such as hydrogen bonds, electrostatic bonds and Van der Waals. All of these may be strengthened in an alkaline environment.

4.5.7 Temperature studies.

Thermal characteristics of *C. gallica* laccase were also investigated. Figure 38 shows that for *C. gallica* laccase the maximum enzyme activity was slightly higher than 65°C. The highest enzyme activity for *T. versicolor* was between 55 °C-60°C. This corresponds well with published values of 60°C for *T. versicolor* laccase (Call and Mucke, 1997). The enzyme rapidly lost its activity at temperatures greater than its optimum. The range for other fungal laccase is between 30 °C-60°C. None of these are thermophilic fungi. A thermophilic fungus has its highest laccase activity between 50 °C-60°C (Fukushima and Kirk, 1995). As the temperature increases beyond its optimum, the laccase loses activity due to heat denaturation of the enzyme. However the 'optimum' temperature of an enzyme is an ambiguous term. It is a compromise between increased activity of the enzyme due to thermal activation and denaturation of the enzyme from heat. For this reason the value is a product of the experimental conditions, so comparison with other fungal laccases can be difficult. Laccase of *P. flavido-alba* was shown to have an optimum activity at 30°C (Perez et al.,

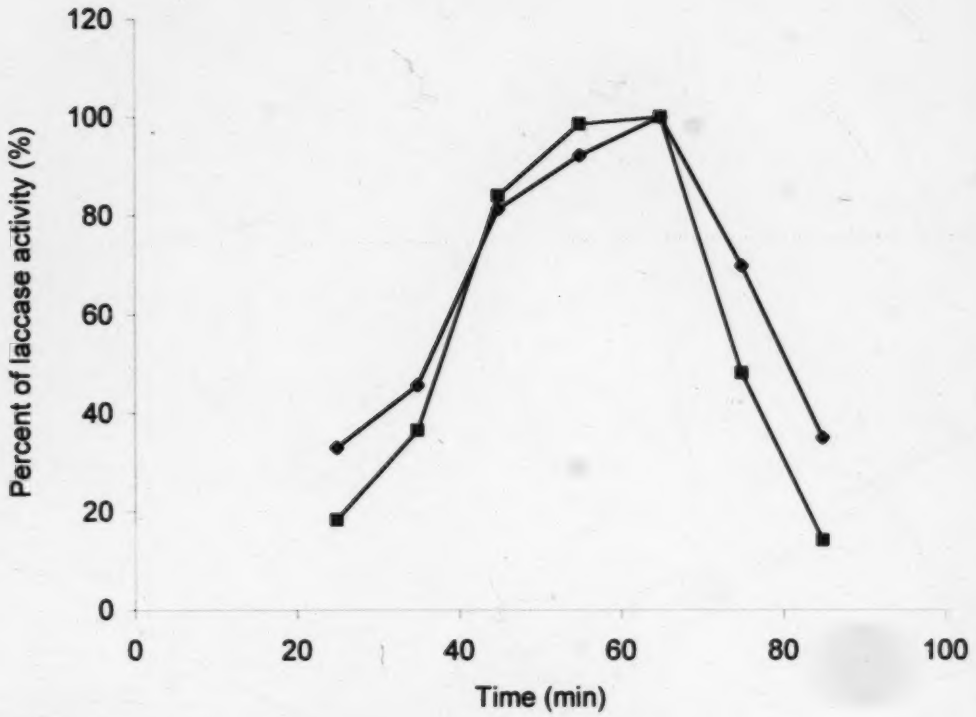


Figure 38. Effect of temperature on purified laccase activity

—◆— *C. gallica* —■— *T. versicolor*

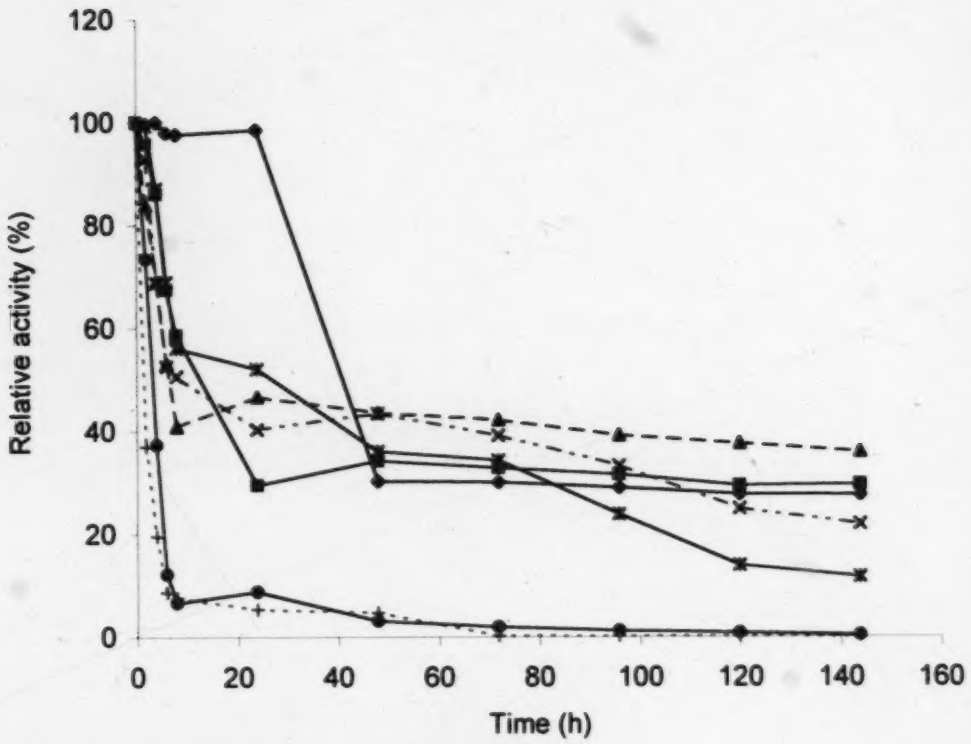


Figure 39. Thermal stability of *C. gallica* laccase

25 oC 35 oC 45 oC 55 oC
65 oC 75 oC 85 oC

1996) while for *P. eryngii*, the highest activity was found at 50°C (Munoz et al., 1997).

The temperature stability of *C. gallica* laccase was also investigated. The laccase was stable at 25°C for 30 h but was reduced to ~30% activity after 45 hours. Temperatures above room temperature caused rapid decreases in activity. For 35°C-65°C there was an immediate rapid loss of activity within 10 h but then activity leveled off at the same level as 25°C, thereafter remaining constant for 6 days. At 65°C, at 3 days there was a decrease in activity. At 75 and 85°C laccase activity was lost within a few hours. Other laccases have similar thermal stability profiles. *P. cinnabarinus* laccase was very stable below 50°C but was quickly inactivated at higher temperatures (Eggert et al., 1996), *P. eryngii* laccase was stable up to 25°C (Munoz et al., 1997), and *C. gallica* A-241 laccase was stable below 28°C but lost activity at higher temperatures.

That the enzyme is stable at room temperature for up to 24 h is a good characteristic. Many chemical modifications are performed at room temperature for optimum modification instead of at 4°C where enzymes tend to be more stable. As the enzyme is stable at room temperature, chemical modifications performed at room temperature for a lengthy period of time should not affect enzyme activity by denaturation due to thermal problems. Laccases in general are stable over a range of 4°C-25°C. This thermal stability can partially be attributed to the carbohydrate moiety, which can stabilize the conformation of the protein through hydrogen bonds between the carbohydrate and the protein. Most laccase are glycoproteins and have between 15%-20% carbohydrate. This makes the enzymes more robust as seen with the laccase from *C. gallica*.

4.5.8 Laccase inhibition.

The effect of several laccase activity inhibitors was examined with ABTS as substrate at pH 4.0, as seen in Table 7. *C. gallica* laccase activity was reduced by a variety of inhibitors, and completely inhibited by 0.1 mM sodium azide, an inhibitor of metalloenzymes. Dithiothreitol, thioglycolic acid and cysteine were all strongly inhibitory. Dithiothreitol is a reducing agent so it will

Table 7. The effect of putative laccase inhibitors on oxidation of ABTS by *C. gallica* laccase.

Compound	Concentration (mM)	Inhibition (%)
NaN_3	0.1	100
L-cysteine	1.0	100
Thioglycolic acid	1.0	100
Dithiothreitol	1.0	100
Phenanthroline	10	25
EDTA	10	28

give an electron to laccase and therefore it cannot oxidize substrates to acquire an electron for O_2 reduction. Thioglycollic acid is a strong reducing agent and an O_2 scavenger. Laccase specifically uses O_2 as an electron acceptor and if there is none it cannot release its electrons to return to resting state to oxidize other substrates. L-cysteine, another reducing agent like dithiothreitol, is able to bind to the enzyme to stop catalysis via the type 1 Cu. These substances inhibited laccase, as expected. However, EDTA and phenanthroline were not strongly inhibitory. Phenanthroline and EDTA are metalloenzyme inhibitors that chelate iron and other divalent cations such as Cu and reportedly show variable inhibition of laccase. By chelating the Cu, the catalysis of the enzyme is limited. It is possible that access of the inhibitors to these Cu atoms is limited and therefore they do not totally inhibit the laccase. They may not bind strongly to the Cu atoms or may only bind to some of the Cu atoms in some of the enzyme present, partially inhibiting laccase. This laccase is inhibited by many anions, which are able to interact with the Cu of the enzyme. It is clear from this inhibition pattern that the laccase from *C. gallica* functions in a similar manner to other laccases.

4.6 Chemical modification of laccase.

With the aim of changing the catalytic behavior of laccase, the enzyme was chemically modified with, either PEG groups or methyl groups. The laccase that was modified with PEG was determined to have 84% of its free amino groups modified as determined by the free amino group assay based on the lysine content of *T. versicolor* laccase (Call and Mucke, 1997). This was termed PEG-laccase. A second PEG modified laccase was prepared with 45% of its free amino groups modified. This was termed ½-PEG-laccase.

The laccase was modified with methyl groups and named Met-laccase. . The boron trifluoride (BF_3) in methanol reagent methylated any free highly reactive carboxyl groups including the C-terminus. The 50% BF_3 solution had a very low pH value and when it was mixed 1:1 with dimethylformamide according to the method of Tinoco and Vazquez-Duhalt (1998), the enzyme was denatured immediately, as indicated by the loss of blue color of the enzyme and bubbling in

the flask. Enzyme activity tests were performed and there was no laccase activity remaining. Therefore, the reagent solution was adjusted to a pH more suitable for the laccase. The BF_3 reagent was diluted to 1% with methanol and when added 1:1 to dimethylformamide the enzyme was still active after the reaction has taken place for six hours. There was no readily available assay to determine the number of free carboxyl groups remaining after modification. The way the modification was judged a success was to look at the specific activity. The modification was judged successful after no apparent loss in color and activity determination showed, as seen in Table 8, the specific activity increased.

One problem with this modification was the poor solubility of dried enzyme in dimethylformamide, which can lead to substrate and product mass transfer problems. The enzyme modification reaction had to be performed in the abscence of water so there was no water present to aid in solubilizing the enzyme. Even after modification with methyl groups there was precipitated protein in the solution and a substantial loss of protein. A possible way to enhance such a modification is to first produce an enzyme that is soluble in organic solvents and then perform methylation. This can be accomplished by using PEG-laccase. In the literature this has been performed successfully (Tinoco and Vazquez-Duhalt, 1998).

4.6.1 Spectral characteristics of chemically modified laccase.

The catalytic behaviour of the modified enzymes must be characterized preliminary to assessing their ability to oxidize PAHs. One of the most important features of an enzyme is its catalytic site. For laccase, the Cu center is important and this portion of the enzyme should not be adversely affected by the chemical modification. The scan for unmodified laccase showed a peak at 606 nm and a shoulder at 330 nm. The scan for PEG-laccase showed a similar profile to that of laccase. There was a shoulder at about 350 nm and there was a peak at 607 nm.

Table 8. Specific activity of methylated laccase preparations.

Trial	Specific activity of native laccase	Specific activity of met-laccase
1	4553	2.13
2	4553	5.32
3	4553	5421

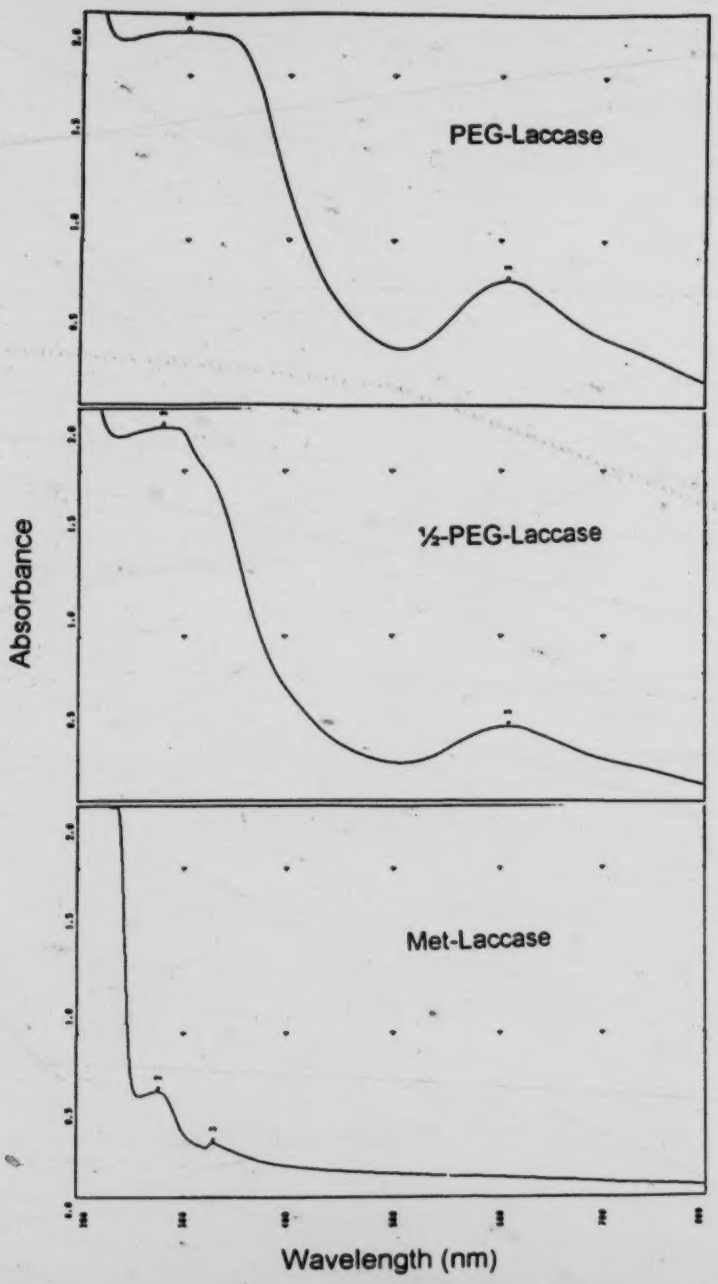


Figure 40. UV-visible spectra of chemically modified laccases. PEG-laccase and ½-PEG-laccase both display peaks at about 600 nm and shoulders at 350 nm, similar to laccase. Met-laccase displays a peak at 350 nm but not at 600nm.

For the same amount of protein, there appeared to be a slightly higher peak at 607 nm for PEG-laccase than for laccase, the extinction coefficient increased. The ½-PEG-laccase displayed a scan similar to laccase. This indicates that the Cu site was unaffected by the addition of the PEG groups, which indirectly indicates that the active site was not affected by the modification.

Comparison of Met-laccase with native laccase indicates a major change in the spectrum. There is a strengthening of the peak of the Cu type 3 or the shoulder at 330 nm but there was no noticeable peak at the blue Cu or at 610 nm. This indicates that there was some change in the Cu content or some effect from the modification. A chemical modification of the active site could lead to adverse changes in catalytic activity of the enzyme. Without a type 1 Cu site there is no mechanism to oxidize a substrate so the activity of the laccase would be severely limited.

4.6.2 Activity of chemically modified laccase in organic solvent.

There are two main factors that must be considered when optimizing an enzyme for application in organic solvents: the activity and stability of the catalyst. The activity of the modified enzymes in organic solvents was considered first using the oxidation of ABTS. Organic solvents have been shown to denature enzymes by stripping away the protective hydration shell. It is thought that the addition of chemical groups will eliminate this process. The objective of the chemical modification is to enhance the hydrophobicity of the enzyme making it more soluble, active and stable in organic solvents. Laccase has been shown to retain ~90% of activity in 15% acetonitrile (Pickard et al., 1999). The activities of the laccase preparations were assayed in the presence of different levels of acetonitrile and were found to retain most of their activity at 15% acetonitrile. The most divergent trend in the data was observed at 30% acetonitrile: laccase retained about 35% of its activity but PEG-laccase and ½-PEG-laccase retained 70% of their activity.

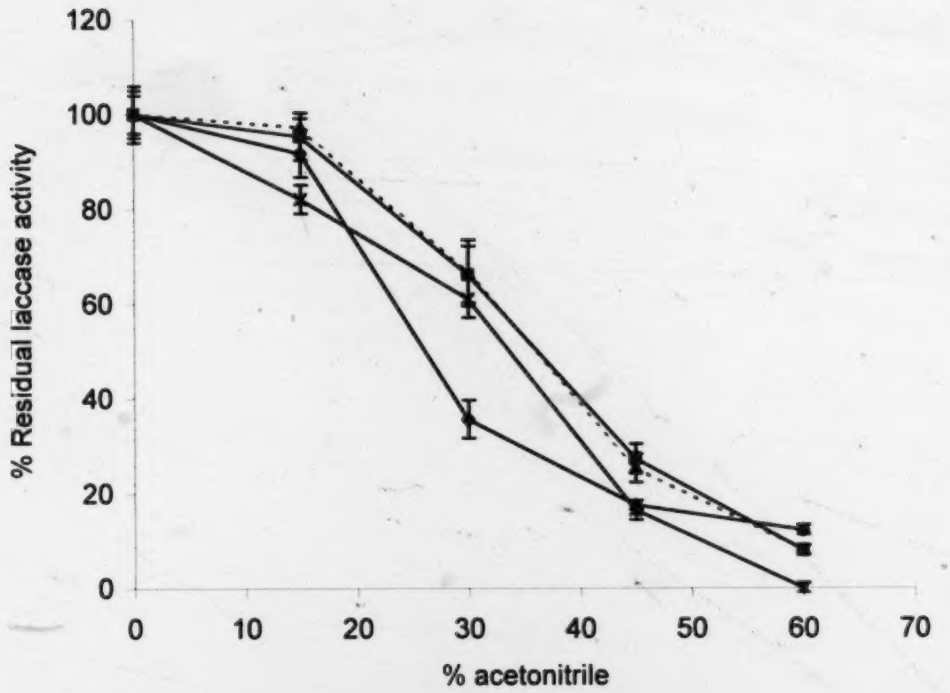


Figure 41. Activity of purified laccase and chemically modified laccases in different amounts of acetonitrile

—◆— laccase —■— PEG-laccase ··▲·· 1/2-PEG-laccase —✱— Met-laccase

The Met-laccase activity at 30% acetonitrile was slightly lower than the PEG-laccases but was significantly higher than for native laccase. At 45% acetonitrile there was between 20 – 35% activity remaining in laccase and the chemically modified laccases. At 60% acetonitrile activity there was no appreciable laccase activity for any form of the enzyme. However the data shows that one point at 30% acetonitrile for PEG-laccase is much lower than the other enzymes tested. The standard deviation of triplicate flasks indicates that the data points do not overlap but the experiment could be redone to confirm the data.

The activity of the laccase was affected by the addition of PEG and methyl groups. Modification of laccase with PEG seems to increase activity at levels up to 45% acetonitrile but methylation seems to decrease activity at higher levels of organic solvent. It is possible that the PEG groups are affording more protection to the enzyme, allowing it to retain its water shell and hence its active conformation. The PEG allows more water to be retained closer to the protein, which will not allow the organic solvent to disrupt the intricate network of bonds holding the enzyme in its active conformation. The modification of the enzyme by the addition of PEG may have enhanced its activity in acetonitrile. PEG-Laccase may show increased activity in higher acetonitrile concentrations due to the fact that they are more soluble and will not precipitate, unlike native laccase.

4.6.3 Stability of chemically modified laccase in organic solvent.

The stability of the enzyme is another important consideration as the enzyme can only be useful in an organic solvent environment if it is stable. The stability of laccase in organic solvent varied with the modification. The stability differences in the different laccases were evident as the amount of acetonitrile increased. At 15% acetonitrile the PEG-laccase and ½-PEG-laccase were slightly more stable than native laccase and Met-laccase. At 30% acetonitrile the stability differences became more evident. After 30 min there was about a 20% difference in the activity of PEG-laccase and native laccase. It is evident that the laccase is being stabilized by the addition of PEG-groups to its surface in the presence of organic

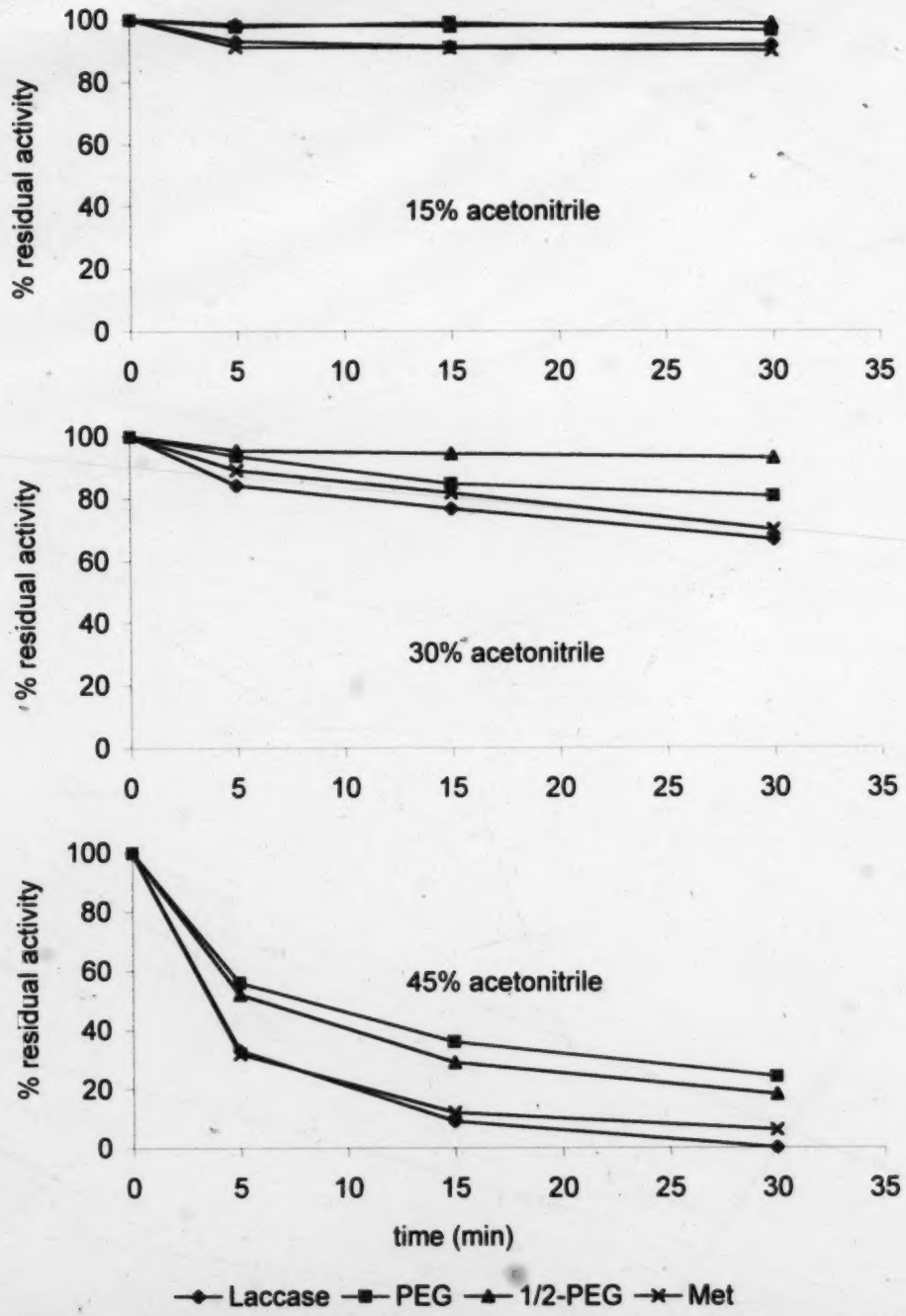


Figure 42. Stability of purified laccase and chemically modified laccases in acetonitrile over a 30 minute time period.

solvents. The trend continues when 45% acetonitrile is added. There is a 40% difference in the activity between PEG-laccase and native laccase after 30 min. In the presence of 60% acetonitrile all activity was lost within a few minutes.

The addition of PEG groups to laccase seemed to have a moderate stabilizing effect on the enzyme, up to 45% acetonitrile compared to native laccase. PEG groups are both hydrophilic and hydrophobic and will help maintain the conformation of the enzyme by maintaining a suitable hydration layer around the enzyme in the presence of denaturing organic solvents such as acetonitrile.

The number or amount of PEG groups does not seem to have much of an effect on the stability. The addition of half as many PEG groups maintains a similar stability pattern to that of the PEG-laccase. It seems that there is a threshold for the number of PEG groups needed for maintaining the hydration shell to stabilize the conformation of the enzyme and this threshold is below the amount of ½-PEG-laccase but further tested would be required for a definitive answer.

The addition of methyl groups does not have the same effect. There is no stabilization of the native laccase by the methylation of the enzyme. These moieties are not as hydrophobic as PEG groups and do not have hydrophilic qualities to help maintain a hydration shell around the protein. In previous papers the stability of lignin peroxidase that had been chemically modified were not improved in the presence of organic solvent (Tinoco and Vazquez-Duhalt, 1998).

The effect of the amount of water on Km and Vmax is most notable when water content is less than 10%, as documented for laccase from *T. versicolor*. There were marked decreases in Km and Vmax as water amount decreased (Van Erp et al., 1991). However the conditions being tested in this research are at a minimum of 40% water so solvent effects on Km and Vmax do not contribute to the effects of the chemical modification. The effect of increasing the activity by adding PEG groups is solely due to that addition.

4.6.4 Substrate specificity of native and chemically modified laccase.

The activity and stability of laccases were affected by the addition of chemical groups. It is thought that the catalytic activity of the enzyme would also be affected. The substrate specificity of native laccase and chemically modified laccase were tested with a range of substrates. Among the compounds oxidized by the enzyme are substituted phenols and aromatic amines. The influence of the nature and number of aromatic-ring substituents on the kinetic constants was considered in the case of substituted phenols. Kinetic parameters were determined at the optimum pH for *C. gallica* laccase, as seen in Table 9. The oxidation rate, affinity and catalytic efficiency revealed a wide substrate range.

ABTS and 2,6-dimethoxyphenol both had higher catalytic efficiencies and oxidation rates than any other substrates but the Km for 2,6-dimethoxyphenol was one of the highest. Vanillic acid, containing a carboxylic acid group, had a 10 fold higher catalytic efficiency than vanillin. The oxidation rates were similar but the Km values were significantly different as the affinity for vanillic acid was greater. For *p*-methoxyphenol, 4-aminophenol and catechol the enzyme had similar catalytic efficiencies. The oxidation rates for *p*-methoxyphenol and catechol were similar but 4-aminophenol was slightly lower. However the affinity for 4-aminophenol was higher (lower Km value) than the affinities for *p*-methoxyphenol and catechol. Addition of a second methoxyl group, as in 2,6 dimethoxyphenol, showed an increase in the catalytic efficiency by 10,000 fold over *p*-methoxyphenol. The oxidation rate was much higher for 2,6-dimethoxyphenol than for *p*-methoxyphenol but the affinity was much lower for 2,6-dimethoxyphenol. Syringaldazine had the lowest catalytic efficiency. In comparison to syringaldehyde there was no indication that an increase in methoxy substituents increased catalytic efficiency. The oxidation rate was much lower for syringaldazine and the Km was much higher than for syringaldehyde. There was no oxidation of tyrosine.

It is clear that the laccase from *C. gallica* has a wide substrate range as it non-specifically oxidizes a variety of substrates. This is not unexpected as most

Table 9. Kinetic constants for substrates of laccase and PEG-Laccase.

Substrate	Laccase			PEG-Laccase		
	Km (μM)	Oxidation rate Kcat (min^{-1})	Catalytic efficiency Kcat/km	Km (μM)	Oxidation rate Kcat (min^{-1})	Catalytic efficiency Kcat/km
ABTS	0.43	2.2×10^{8}	4.9×10^{5}	0.25	5.0×10^{9}	1.9×10^{7}
vanillin	14	2.2×10^{5}	1.6×10^{1}	8.2	7.3×10^{6}	8.9×10^{2}
vanillic acid	2.9	4.8×10^{5}	1.6×10^{2}	6.1	9.5×10^{6}	1.6×10^{3}
ρ-methoxyphenol	2.6	7.7×10^{5}	2.9×10^{2}	2.8	1.9×10^{8}	6.6×10^{4}
4-aminophenol	0.63	1.7×10^{5}	2.7×10^{2}	1.4	2.8×10^{8}	2.0×10^{5}
2,6-dimethoxyphenol	3.5	3.6×10^{5}	1.0×10^{7}	1.6	6.1×10^{9}	3.9×10^{6}
catechol	3.1	7.7×10^{5}	2.4×10^{2}	3.8	8.5×10^{6}	2.2×10^{3}
HBT	0.54	4.3×10^{5}	7.7×10^{2}	3.9	8.9×10^{6}	2.3×10^{3}
syringaldazine	2.8	2.6×10^{0}	9.4×10^{-4}	1.3	1.4×10^{4}	1.1×10^{1}
syringaldehyde	0.40	1.6×10^{6}	3.9×10^{3}	0.14	2.3×10^{9}	1.8×10^{6}
tyrosine	ND	0	0			

fungal laccases are non-specific in terms of their reducing substrate. However the enzyme differs in its ability to oxidize these substrates. The affinity, oxidation rate and the catalytic efficiency of the laccase can be affected by substitution of the substrate. The differences in the ability of the laccase to utilize substrate can be attributed both to the binding of the substrate as seen with the Km, affinity, and to catalysis of the enzyme as seen by the oxidation rate. The contribution of each kinetic parameter is a property of each substrate. The better the oxidation rate and the higher the affinity the better the substrate. Kcat reflects a combination of these values, which displays the overall effectiveness of the enzyme. There is a balance between affinity of the substrate and oxidation rate of the substrate. Addition of a carboxylic acid to the aromatic ring increased affinity and catalytic efficiency but the oxidation rate remained constant as seen when comparing vanillic acid and vanillin. Increasing the number of methoxyl substituents also increase the catalytic efficiency and oxidation rate, as seen when comparing 2,6-dimethoxyphenol and p-methoxyphenol but not the affinity. In general, increasing the number of substituted groups increased the catalytic efficiency. Comparing the kinetic constants obtained for 4-aminophenol, *p*-methoxyphenol and catechol it is seen that the catalytic efficiency of laccase was similar for phenols, amines and hydroxyls substituted on phenol. All of the substituents are electron donor groups and this laccase does not have a preference. Chefetz et al., (1998), also found similar substrate patterns for *C. thermophilium* laccase. The nature and substitution of the phenolic ring affected the kinetic parameters of the *C. gallica* laccase. It can be concluded from these experiments that ABTS and 2,6-dimethoxyphenol are preferred substrates. *C. gallica* A-214 also utilized ABTS and 2,6-dimethoxyphenol as superior substrates (Calvo et al., 1998).

It is worth noting that the kinetic parameters for syringaldazine were determined in the presence of 5% ethanol due to low solubility of the substrate. It was determined that ethanol does not have an adverse affect on laccase activity at this concentration using ABTS as substrate. The lack of activity of this

enzyme for syringaldazine is unusual in that this is a good substrate for other laccases. In fact this substrate is used to determine laccase activity of cultures for some fungi (Palmieri et al., 1993). For *P. ostreatus*, the pH optimum was determined to be between pH 3.0 to 3.5 with ABTS but was pH 6.0 to 7.0 for syringaldazine. This difference was proposed to be due to the different role of substrate protonation in the reaction mechanism (Palmieri et al., 1993). As these substrates were being tested at the optimum pH of laccase it may not be a good pH for syringaldazine, as this substrate may not be in the proper form for use by *C. gallica* laccase.

It recently has been shown that significant differences in redox potential exist among fungal laccases (0.5 to 0.8 V) and that the Vmax values of reactions catalyzed by these enzymes are positively correlated to differences in redox potential between laccase and substrates (Munoz et al., 1997; Xu, 1996). It has been shown that *P. eryngii* laccase oxidized phenolic compounds with a redox potential of $\leq$ 0.6 V (Munoz et al., 1997). Since all of the compounds with known redox potential tested have a value lower or equal to this limit, it is not surprising that all substrates were oxidized.

In laccase catalysed multi-electron phenol oxidation, the first electron transfer from phenol to laccase is rate limiting and is governed by the redox potential difference between the substrate and laccase type 1 Cu (Xu, 1996). Vanillic acid is used more efficiently than vanillin. It has a lower redox potential than vanillin. The lower the redox potential of the substrate, the greater the difference between substrate and laccase, which means a greater catalytic efficiency. Unfortunately, this theory does not hold true for all substrates. Comparing *p*-methoxyphenol and 4-aminophenol, there is a 4-fold difference in redox potential but little difference in catalytic efficiency. It is clear that not just redox potential or the electronic contribution of a substituent has a role in determining substrate specificity.

There was no activity towards tyrosine so there is no *o*-hydroxylation activity which is characteristic of catechol oxidase, a tyrosinase, confirming that

Table 10. Kinetic constants for ½-PEG-laccase and Met-laccase

Substrate	½-PEG-Laccase			Met-Laccase		
	Km (μM)	Oxidation rate Kcat (min^{-1})	Catalytic efficiency Kcat/km	Km (μM)	Oxidation rate Kcat (min^{-1})	Catalytic efficiency Kcat/km
ABTS	0.10	2.9×10^{8}	2.8×10^{6}	0.44	7.0×10^{5}	1.6×10^{2}
vanillin	5.8	3.5×10^{6}	5.9×10^{2}	3.7	1.8×10^{6}	4.8×10^{1}
vanillic acid	2.1	9.1×10^{6}	4.4×10^{3}	7.6	6.9×10^{5}	9.1×10^{1}
p-methoxyphenol	3.5	1.4×10^{8}	3.9×10^{4}	2.9	2.4×10^{5}	8.2×10^{1}
4-aminophenol	1.9	1.1×10^{8}	5.7×10^{4}	1.7	3.0×10^{5}	1.8×10^{2}
2,6-dimethoxyphenol	1.1	2.3×10^{9}	2.0×10^{6}	0.28	3.2×10^{5}	1.1×10^{3}
catechol	1.1	6.5×10^{6}	5.9×10^{3}	2.0	7.2×10^{4}	3.6×10^{1}
HBT	6.3	3.8×10^{6}	6.1×10^{2}	1.1	1.7×10^{5}	1.6×10^{1}
syringaldazine	4.5	2.9×10^{4}	6.5×10^{0}	1.2	2.3×10^{3}	1.9×10^{0}
syringaldehyde	0.24	1.6×10^{8}	6.6×10^{5}	0.39	7.2×10^{5}	1.8×10^{3}

the enzyme being studied is a laccase. It has been observed that the catalytic Cu site of laccase is highly conserved among fungal laccases. This extends to the sequence of laccase genes (Eggert et al., 1997). However all laccases studied have differing substrate specificity. The laccase from *C. gallica* has been demonstrated to have a unique substrate specificity. Not only must part of the active site be involved in substrate specificity, but other amino acid residues in the enzyme could participate. Whether this is due to binding differences between enzymes or the redox potential of the enzyme it is not known. There may be areas of amino acid variability between laccases near the active site that account for the differences in binding or redox potential. More laccase gene sequences will help develop a better understanding of the structure function relationships that govern substrate specificities and functions of laccase in different biological systems.

The substrate specificity was not altered by the chemical modification of laccase. All substrates tested were oxidized by all chemically modified enzymes as seen in Table 10. However the catalytic efficiency was significantly altered. The catalytic efficiency of substrates was increased by up to 1000 fold for PEG-laccase compared to native laccase. A dramatic 1000 fold increase was seen for 4-aminophenol. There were significant differences between syringaldazine and syringaldehyde. The catalytic efficiency for syringaldehyde was 10,000 fold higher than syringaldazine. The oxidation rate was lower and the Km was higher for syringaldazine. Unlike native laccase, PEG-laccase showed considerable difference in preference for particular functional substituents. The catalytic efficiency 4-aminophenol was 10 fold higher than the value for *p*-methoxyphenol which was 10 fold higher than the value for catechol. *p*-Methoxyphenol had a higher oxidation rate than the other two and a lower Km value.

For ½-PEG-laccase, the catalytic efficiencies of substrates were lower than for PEG-laccase but higher than for native laccase with some variability. Similar trends were observed among compounds for comparison of the effect of functional groups. Vanillic acid had a catalytic efficiency that was 10 fold greater

than vanillin. The oxidation rate was higher and the Km was lower for vanillic acid. Syringaldazine had a catalytic efficiency lower than for syringaldehyde. Catechol's catalytic efficiency was 10 fold lower than 4-aminophenol and *p*-methoxyphenol. The addition of a second methoxyl group increased the catalytic efficiency by 10 fold. These trends are similar to PEG-laccase.

The increase in catalytic efficiency can be due to several factors: including an increase in the affinity (lower Km) of the laccase for a substrate or an increase in the oxidation rate. The affinity of the enzymes was affected in different ways by the chemical modification of the enzyme. In some cases the affinity decreased but in most cases affinity increased. In all cases the oxidation rate increased. It appears that the chemical modification may have affected the active site to allow for easier substrate binding and catalysis. As well the specificity of the enzymes has been altered in terms of functional groups. This may be due to a change in conformation of the protein with the addition of PEG that effectively changes the active site in a favorable manner. This may indicate better binding of the enzyme due to a slightly larger active site or more exposed ligands that bind to the substrate to allow for electron abstraction. The increase in affinity seen by the addition of PEG groups could indicate that there is an increase in hydrophobicity of the active site. The affinity of more hydrophobic substrates increased whereas the affinity of more hydrophilic substrates decreased this is best exemplified by vanillin and vanillic acid. It is possible that the increase in hydrophobicity is related to the change in catalytic behavior. This was postulated as a reason for the increase in catalytic behavior of chemically modified cytochrome C (Tinoco and Vazquez-Duhalt, 1998). Overall, there is an improvement in the catalytic efficiency of PEG-laccase over the catalytic efficiency of native laccase.

The Cu content of the enzyme was not affected by the addition of PEG groups as indicated by the spectrum, so the change in substrate specificity cannot be attributed to a change in the Cu of the active site. It is possible that there is a conformational change that stabilizes the enzyme and also slightly

changes the conformation of the active site which, is able to increase the catalytic efficiency of the enzyme. The chemical modification of lignin peroxidase with aromatic moieties affected the kinetic constants. However correlation could not be obtained between the substrate hydrophobicity, the type of chemical modification or the kinetic constants. The reason for the kinetic changes by chemical modification of the enzyme was unknown (Vazquez-Duhalt et al., 1995). The reasons for the kinetic changes for this enzyme were not evident in the research.

The substrate specificity was also tested for Met-laccase. Met-Laccase displayed much lower catalytic efficiencies for all substrates compared to all other forms of laccase but similar trends were observed between the individual substrates. The Cu content of Met-laccase was affected by the reaction causing the addition of the methyl group. The type 1 Cu signal was absent from the absorption spectrum indicating that the oxidation of the substrate should not be possible as the type 1 Cu is involved in electron capture from the substrate. The lower catalytic activity of the enzyme indicates that the type 1 Cu is affected. The small amount of catalysis can be an indication that not all of the enzyme was affected by the chemical modification. During catalysis the Cu atoms in laccase are in their oxidized state. But if the chemical modification reduced the Cu atom then it would no longer appear in the spectrum because the charge transfer from cysteine to Cu would not longer occur. If not all of the Cu is in its reduced state then some catalytic activity will remain.

From the above it is clear that chemical modification with PEG-groups enhanced the activity and stability of laccase over the native laccase. However the addition of methyl groups has adverse affects on the ability of the enzyme to oxidize substrates and did not enhance the stability in organic solvents.

4.6.5 *In vitro* oxidation of PAHs by laccase and modified laccase.

Laccase from *C. gallica* is known to metabolize some PAHs (Pickard et al., 1999) and mineralization experiments have indicated that the laccase is involved in degrading PAHs *in vivo*. We therefore examined the *in vitro*

Table 11. Rates of oxidation of PAHs by *C. gallica* laccase and PEG-laccase with various mediating substrates.

PAH	λ (nm)	Ionization potential (eV)	Reaction rates (k) Laccase				Reaction rates (k) PEG-Laccase			
			No mediator	ABTS	HBT	ABTS + HBT	No mediator	ABTS	HBT	ABTS + HBT
Anthracene	225	7.55	0.857	0.539	0.886	1.59	0.788	4.29	9.35	14.7
Phenanthrene	250	8.03	1.66	1.49	1.80	1.27	1.63	1.93	1.92	1.22
Chrysene	250	7.80	0.0051	0.0298	0.001	0.0038	0.0269	0.0589	0.0757	0.0137
Pyrene	236	7.72	0.0276	0.276	0.691	0.829	0.276	0.276	0.263	0.318
Acenaphthene	226	7.7	1.38	1.42	13.5	1.34	1.09	1.13	10.7	6.01
9-methylanthracene	253	7.23	1.26	1.16	2.13	1.01	1.35	2.00	3.84	2.00
2-methylanthracene	247	7.42	0.497	12.9	10.2	21.0	0.912	2.67	0.622	39.3

metabolism of PAHs by chemically modified *C. gallica* laccase to determine if this modification enhanced PAH metabolism. The solubility of PAHs in water is low, which leads to mass transfer problems for their metabolism, but the addition of an organic water miscible solvent such as acetonitrile increased the solubility of PAHs and the presence of this organic solvent in low concentration was not harmful to the activity and stability of chemically modified laccases.

The oxidation of seven PAHs was tested using native laccase, PEG-laccase, ½-PEG laccase and Met-laccase as seen in Tables 11 and 12. The results were variable and depended on the PAH tested and the form of the enzyme. Interestingly, with the addition of PEG it was possible to alter the substrate specificity. Of the seven substrates tested the native laccase was able to oxidize four substrates whereas the PEG-laccase and ½-PEG-laccase was able to oxidize five substrates. Methylation did not have a positive effect on the *in vitro* metabolism of most PAHs compared to native laccase, except for 9-methylanthracene.

Since the phenanthrene oxidation rates were unchanged by addition of mediating substrates, or chemically-modified laccases, this oxidation may well be nonenzymatic.

Chemical modification of the enzyme did seem to increase the substrate range of the laccase. The PEG-laccase was able to oxidize five PAHs compared to the four for native laccase. The ability to utilize larger PAHs could be due to steric factors. The addition of PEG groups could have increased the size of the active site to allow the binding of the larger PAHs or by making the laccase more hydrophobic, substrate availability may have been enhanced. Enlarging the scope of the PAHs studied would lead to an answer to this question. Expansion of the substrate specificity is not novel: a cytochrome C with a double modification (PEG on free amino groups and methyl esters on carboxylic groups) was able to oxidize 17 aromatic compounds of the 20 tested while the unmodified protein was only able to oxidize 8 compounds. It was determined that it was probably the PEG groups that caused the change in substrate specificity, as the

Table 12. Rates of oxidation of PAHs by ½-PEG-laccase and Met-laccase with various mediating substrates.

PAH	Reaction rate (k) ½-PEG-Laccase				Reaction rate (k) Met-Laccase			
	No mediator	ABTS	HBT	ABTS + HBT	No mediator	ABTS	HBT	ABTS + HBT
Anthracene	0.276	1.08	0.84	9.91	0.0553	0.0829	0.165	1.20
Phenanthrene	0.995	0.801	0.705	0.373	0.843	0.359	0.553	0.622
Chrysene	ND	ND	ND	ND	ND	ND	ND	ND
Pyrene	0.0829	0.0967	0.0967	0.124	0.289	0.0553	0.124	0.111
Acenaphthene	1.08	1.44	8.5	5.2	1.08	1.13	1.71	1.79
9-methylanthracene	1.24	1.97	2.99	1.96	2.65	2.35	7.77	11.8
2-methylanthracene	0.428	2.97	0.981	39.2	0.387	0.539	0.691	6.1

fully methylated cytochrome C showed the same substrate pattern as the unmodified cytochrome C (Tinoco and Vazquez-Duhalt, 1998). It was possible to change both the catalytic activity and substrate specificity of the cytochrome C. This indicates that chemical modification of an enzyme could be useful for designing new enzymes with environmentally useful properties.

Study of the native laccase showed that some PAHs were transformed by laccase, but larger PAHs such as chrysene and pyrene were not significantly degraded. The addition of mediating substrates, ABTS and HBT, to the mixture had variable effects on the degradation of PAHs. The degradation of 2-methylanthracene with ABTS was significantly increased but in most cases activity was unchanged or was reduced with the other PAHs tested. Addition of HBT increased or maintained laccase activity with all PAHs. Both acenaphthene and 2-methylanthracene oxidation were positively affected by the addition of HBT. The addition of both mediating substrates also caused mixed results, both increasing and decreasing the oxidation rate of the PAHs. 2-Methylanthracene showed a 42-fold increase in the rate constant whereas most other PAHs showed a 1 or 2 fold increase. Controls with boiled enzyme did not show significant rates of reactions.

The ionization potential does not seem to correlate with the ability of laccase to oxidize PAHs. The ionization potential was used for PAHs as redox potentials were unavailable for the compounds being studied. Pyrene, a PAH with a low ionization potential, is not oxidized by laccase. The first order rate constants for these reactions are not clearly related to the ionization potential of the PAHs. A similar trend was observed by Pickard et al. (1999) when laccase from *C. gallica* was tested for the oxidation of several PAHs. In a study by Collins et al. (1996), the IP value of the PAHs appeared to affect its susceptibility to oxidation by laccase. Anthracene and benzo[a]pyrene, both of which had IP values of < 7.45 eV, could be oxidized by laccase while fluorene and phenanthrene, which have IP values of > 8.0 eV, remained unoxidized. A later, more extensive study showed a discrepancy in the correlation between IP values

and the oxidation of PAHs by laccase and laccase/mediating substrate systems. PAHs with IP below ~7.45 eV revealed a very good oxidation of over 48%. Above this IP value, no uniform correlation was found. Acenapthene was very well degraded, but it has an IP of 7.8 eV. These findings are not surprising since PAHs are a large group of polycyclic aromatic compounds with different structural variations and differing chemical reactivity (Majcherczyk et al., 1998).

The PAHs were more affected by the addition of HBT by itself. When HBT is oxidized it produces a more reactive intermediate than when ABTS is oxidized. ABTS cations are more stable than the nitroxyradical, which reacts rapidly with various aromatic compounds. Nitroxyradicals are more potent electrophiles that easily abstract allylic hydrogen (Bohmer et al., 1998). Therefore it seems likely that the nitroxyradical oxidizes PAHs better than ABTS as seen in the results.

In order for ABTS to work as a mediating substrate, it must be oxidized to ABTS $^{2+}$. This species must be present for mediation to occur for the oxidation of nonsubstrate compounds of laccase such as PAHs. As observed, the reaction mixture turned a purple-red color from an intense blue color. This is indicative of the ABTS $^{2+}$ species being present which meant that oxidation of nonphenolic compounds could occur. The presence of ABTS $^{2+}$ was not observed until later in the reaction time indicating a delay. This could have been why ABTS was not as effective a mediating substrate as HBT as the oxidizing species was not formed as quickly or as efficiently for ABTS as for HBT.

There are two main parameters that can affect the oxidation rates of compounds when mediating substrates are required. First, the enzyme can be inactivated by a mechanism that is not completely understood. The radicals that are formed upon the oxidation of compounds could be deleterious to the enzyme causing loss of activity. The mediating substrate only works when active enzyme is present. In this case, the radical produced from ABTS by laccase could be deleterious to the enzyme. Another important parameter is the Kcat of the laccase for the laccase mediating substrate. The Kcat for ABTS is greater than that of HBT for *C. gallica* laccase but HBT works better as a mediating substrate,

which is unexpected. From the data observed, HBT is the better mediating substrate for *C. gallica* laccase.

The addition of both mediating substrates does not have a synergistic effect on oxidation of PAHs, as was previously shown (Pickard et al., 1999). Anthracene was the only PAH that revealed a synergistic effect when both ABTS and HBT were added to the reaction mixture in this study. This is similar to a result by Pickard et al. (1999) where anthracene oxidation with mediating substrates was shown to have a synergistic mechanism. It appears that the presence of ABTS is inhibitory to the action of HBT when ABTS itself does not affect oxidation rate. This may be because *C. gallica* laccase has been shown to greatly prefer ABTS as a substrate over HBT indicating that HBT will not be used preferentially. It appears that the mechanism that causes mediation is different in different conditions.

Further comparison of the data in this study compared to a study by Pickard et al. (1999) shows other discepancies. The rate constant for 9-methylanthracene was substantially higher (over 100 fold) in the Pickard et al. (1999) study than in ours. The reverse was found for 2-methylanthracene where the rate constant was found to be ~ 9 fold higher in this study. These differences further exemplify that experimental conditions are an important consideration when studying these systems.

PEG-laccase was also tested for its ability to transform PAHs. Incubation without mediating substrates showed that PEG-laccase oxidized the four PAHs similarly to native laccase. However the degradation of chrysene was increased by 5 fold and pyrene was increased by 10 fold. Incubation with either ABTS or HBT increased or maintained the catalytic activity in all PAHs compared to incubation without mediating substrates. Substantial increases were observed for anthracene, a 5 fold increase, and pyrene, a 10 fold increase, when ABTS was added. Even more substantial increases were observed for anthracene, a 12 fold increase, and acenaphthene, a 10 fold increase, when HBT was added. Addition of both ABTS and HBT significantly increased the rate of anthracene

oxidation by 20 fold, increased the rate with 2-methylanthracene by 45 fold , the rate with acenaphthene by 6 fold and the rate with pyrene by 12 fold but all other PAHs were minimally affected by a 1 to 2 fold increase in rate when mediating substrates were added. The reaction rate for phenanthrene was unaffected by the addition of mediating substrates and it was not affected by the modification. The reaction rates of PEG-laccase without mediating substrate were generally the same as for laccase. The addition of mediating substrates was more dramatic when the enzyme was modified with PEG groups than without modification. Controls with boiled PEG-laccase did not show significant rates of reactions but all rates were substantially above these rates.

½-PEG-laccase was tested for its ability to transform PAHs. Incubating without mediating substrates showed that the ½-PEG-laccase did not increase the reaction rate of PAHs. The reaction rate either decreased or remained the same compared to both laccase and PEG-laccase. Incubation with the mediating substrates displayed some significant increases in rate, mainly with anthracene (36 fold increase) and 2-methylanthracene (91 fold increase) in the presence of both mediating substrates. Similar trends were seen with the addition of individual mediating substrates. The reaction rate with phenanthrene was unaffected by the addition of mediating substrates. The reaction rates for ½-PEG-laccase were, in general, lower when compared to laccase or PEG-laccase. Controls with boiled ½-PEG-laccase did not cause oxidation of PAHs. The addition of PEG groups seems to increase the oxidation rate but the number of PEG groups added is an important factor.

Met-laccase, upon incubation without the mediating substrates, displayed reaction rates that were lower than native laccase, the exceptions being 9-methylanthracene and pyrene, but these rates were at most 4 fold higher. The addition of ABTS into the reaction mixture did not significantly enhance the ability of laccase to oxidize the PAHs. The addition of HBT in some case did increase the oxidation rate, namely anthracene, pyrene and 9-methylanthracene were all increased by 2 to 3 fold. In other cases the reaction rate remained the same or

decreased. The addition of both ABTS and HBT significantly increased (15 fold) the reaction rate with 2-methylanthracene, increased the rate with 9-methylanthracene by 5 fold and increased the rate with anthracene by 20 fold over native laccase. All other PAHs were either unaffected by the addition mediating substrates or the rate of reaction decreased. The reaction rate of phenanthrene was unaffected by the addition of mediating substrates. Boiled Met-laccase controls did not show any significant rates of reaction when tested.

Chemical modification of the enzyme appears to have limited effects on the oxidation rate of the PAHs hence it is indicated that the redox character or the hydrophobicity of the enzyme has been modified by the addition of PEG or methyl groups. It was thought that a more hydrophobic enzyme would be more attracted to a hydrophobic substrate and that this attraction would increase with an increase in the hydrophobicity of the enzyme. The presence of organic solvents tends to partition the substrate away from the active site. Increasing the hydrophobicity of the enzyme can be a method to attract the hydrophobic substrate. An enzyme modified with PEG groups is more hydrophobic and should have a stronger attraction to hydrophobic compounds such as PAHs. This is reflected in some of the reaction rates calculated for some of the forms of the enzyme tested. This indicates that the hydrophobicity of the substrate was not the only limiting factor for oxidation. As well, increasing the hydrophobicity of the enzyme allows it to be present, stable and active in a hydrophobic environment that is more suitable for the PAH in terms of solubility. The PAH is at a higher concentration in the solution with organic solvent. The mass transfer limitation seen in an aqueous environment is not as problematic. The hydrophobic nature of the enzyme should attract the hydrophobic compounds which should therefore increase the rate of oxidation in combination with increasing concentration of PAH. There were inconsistent increases in oxidation rates of PAHs by chemically modified enzymes. Initial oxidation studies with known substrates indicate that the active site had become more hydrophobic with PEG-laccase. It would seem reasonable that there should also be an increase in

the affinity of hydrophobic compounds such as PAHs. It was thought that by increasing the surface hydrophobicity of laccase by chemical modification, its activity in organic solvent may be increased. Successes have been found in the literature where the chemical modification of enzymes have increased the catalytic behavior of the enzyme. Lignin peroxidase from *P. chrysosporium* was chemically modified by reductive alkylation with benzyl, naphthyl and anthracyl moieties, which increased the superficial hydrophobicity of the enzyme. These altered the kinetic behavior of the enzyme in acetonitrile with four substrates including carbazole, pinacyanol, pyrene and veratryl alcohol. Benzyl modification of lignin peroxidase increased the catalytic efficiency 2.7 fold for carbazole oxidation. The oxidation rate was increased as much as 2.5 fold with veratryl alcohol. The authors postulated that the increase may be due to the increase in the affinity of the enzyme for hydrophobic substrates (Vazquez-Duhalt et al., 1995). The hydrophobicity of the modification moieties may play a role in this effect. Anthracyl and such groups are more hydrophobic than PEG or methyl which may be a reason increases in oxidation were not observed. The increase in hydrophobicity was not enough to make a difference in oxidation rate of the enzyme towards most hydrophobic substrates.

Substrate partition between the active site and solvent seems to be an important factor for biocatalysis of hydrophobic substrate by lignin peroxidase and cytochrome C. It is thought that this would also be an important factor in similar proteins such as laccase. A change in the hydrophobicity may cause a change in the catalytic behaviour. As seen with PEG-cytochrome C the catalytic efficiency of the protein was 9-fold higher than the unmodified enzyme (Vazquez-Duhalt, 1995). This is true for the PEGylated laccase where there was substantial increases in catalytic efficiency for substrates. The spectrum indicates that the Cu content is not negatively affected by the addition of PEG groups. This does not preclude the addition of PEG groups to other areas of the active site thereby increasing the partitioning of hydrophobic substrate to the active site. It is clear that with the addition of PEG groups, the specificity of the

enzyme changed.

Modification with methyl groups had an adverse effect on the activity of the enzyme. As seen with the substrate specificity the catalytic efficiency of Met-laccase decreased for all substrates. The same general trend was observed for the action of Met-laccase on PAHs. This is a clear indication that methylation of this enzyme does not have positive effects on its activity. As indicated by the spectrum the active site is affected in its Cu atoms. This indicated that methylation or the process of methylation causes inactivation of the enzyme.

Another factor that might affect laccase activity is the ionization potential of the substrates. The chemical modifications may have caused an increase in the redox potential of the enzyme to enhance laccase oxidation of PAHs. Most of the compounds that were oxidized have ionization potentials below 8 eV. The capacity of polycyclic aromatic compounds to form radical cations is related to their ionization potential. Phenanthrene has a high IP of 8.03 eV and is not usually considered a substrate of laccase. The reactions observed here are considered non-enzymic. Pyrene has a lower IP and was not oxidized significantly by laccase. The other PAHs all have sufficiently low IP that their oxidation by laccase was not surprising. There appears to be little correlation between the IP and the ability of laccase to degrade a PAH. Similar results were observed for PEG-laccase. Phenanthrene is oxidized by PEG-laccase but pyrene is also oxidized. All other PAHs were oxidized as expected by their IP. ½-PEG-laccase and Met-laccase oxidation of PAH could also not be correlated with IP.

The effects of two mediating substrates, HBT and ABTS, on the oxidation of PAHs by chemically modified laccases were also investigated. The ability of the laccase-mediating substrates to accelerate oxidation seems to act differently in different systems. The accelerative ability of the laccase mediating substrates, ABTS and HBT, appear to work differently in different systems. There have been many reports of mediating substrates enhancing enzyme activity, but each report had its own trend. For example, veratryl alcohol was oxidized by the laccase of

T. versicolor but, with the addition of HBT, it was accelerated twice as much as with ABTS (Bourbonnais and Paice, 1990). Using another *T. versicolor* laccase, ABTS was more effective, 7 fold, than HBT. It is clear that the stimulatory effects are dependant on the system being studied. The variability seen in the accelerative ability of mediating substrates of chemically modified laccases is not completely unexpected. The reasons behind the variability are not elucidated in this research. In most cases similar trends were seen to that of native laccase in that HBT is the better mediating substrate and the addition of ABTS and HBT is not synergistic. It would therefore seem reasonable that similar mechanisms are responsible for the accelerative ability of mediating substrates for both native laccase and modified laccase.

Modification of laccase structure to increase its Kcat is a way to develop a better laccase mediating substrate system. The chemical modification of laccase with PEG groups increased the Kcat for both mediating substrates tested, ABTS and HBT. For ABTS there was a 50 fold increase in Kcat and for HBT this value was even larger. It was thought that the mediating substrate system would be more effective because of the increase in Kcat, which would effectively increase the amount of radical present. For anthracene, this was certainly the case in that both the mediating substrates were able to substantially increase the reaction rate of PEG-laccase over laccase. This was also true for pyrene, chrysene and 9-methylanthracene. But other PAHs such as acenaphthene and 2-methylanthracene had very different results. For acenaphthene there was no increase in oxidation rate for either mediating substrate alone but when both were present there was an increase in oxidation rate. 2-Methylanthracene had even more confusing results, the reaction rates with each individual mediating substrate decreased when the laccase was PEGylated but the reaction rate increased when both were present. It is clear that the effectiveness of a mediating substrate system is dependant on the laccase, the mediating substrate and the nonphenolic substrate in the system.

The catalytic efficiency of ½-PEG-laccase for ABTS was not a significantly

altered by the addition of PEG groups, only a <10 fold increase was observed. The consequences of this were seen in the oxidation of PAHs where the rates of reaction were not as intensely increased by the addition of the mediating substrates. The exceptions can be rationalized by the fact that the system was complex when mediating substrates were added. Any number of variables could participate in the system including steric hindrance and the electronic characteristics of the substrate. Adding another variable such as the chemical modification could intensify the affects of these contributions to the system so variability of results was to be expected.

4.7 Conclusions.

C. gallica produces high amounts of laccase when grown in bran flakes production medium. *C. gallica* laccase is similar to laccases of other ligninolytic basidiomycetes. It has many of the typical laccase characteristics. It is clear from the data presented that there is the possibility that laccase from *C. gallica* is involved in PAH metabolism. The laccase is a typical laccase with a few properties that are especially useful for biotechnological applications. Its carbohydrate content is a stabilizing factor in its increased temperature stability. As well, it has an extended pH stability range which is advantageous for chemical modification procedures. It appears that chemical modification with PEG and methyl groups had little effect on the oxidation of all PAHs, but the accelerative activity of mediating substrates were shown to be substantial in most cases. The role of *C. gallica* laccase in PAH metabolism should be further studied.

5. Future studies.

5.1 Metabolic pathway studies.

In this study there were no attempts to identify PAH metabolites. It would be prudent in the future to determine the metabolites produced by the *in vitro* metabolism of PAHs by laccase of *C. gallica*, both in its native form and when it is chemically modified. This would lend insight into the mechanism by which the laccase degrades the PAHs and how or if this pathway differs when chemically modified laccases are utilized. A good starting point for metabolite identification would be to look for quinones and hydroxylated compounds as these are common metabolites from PAHs by WRF. Instead of utilizing HPLC to identifying the loss of a parent compound peak it would be fruitful to look at the resulting peaks. Utilizing the disappearance of a peak makes it difficult to decide whether the compounds are being partially degraded, mineralized, absorbed to the fungal biomass or other fates. Other methods such as gas chromatography can be utilized to determine metabolites. As well radiolabelled compounds and TLC couldbe used to identify metabolites.

5.2 Other modifications.

5.2.1 Chemical modifications.

The chemical modifications utilized in this study were only a small sample of the numerous chemical modifications and combinations of chemical types possible. The enzyme modified with PEG can be further modified with methyl groups in a double modification to enhance the catalytic activity of an enzyme. As well, reductive alkylation with aromatic substituents such as anthracyl has previously been performed and has been shown to have substantial effects on the catalytic ability of lignin peroxidase. Another chemical modification that could be performed is trimethylsilyl addition on amino, carboxylic and phenolic groups. This modification can produce insoluble protein, but when performed in conjunction with PEGylation, the protein is soluble. All of these can be assessed for their ability to oxidize PAHs and determine if they function better than native

laccase. There are numerous possibilities for enzyme modification and should be explored for optimum functioning of the native laccase from *C. gallica*.

5.2.2 Immobilization.

Another method for enhancing enzyme activity in a hydrophobic environment is immobilization, for example onto a membrane. It is thought that immobilization helps the enzyme maintain its active conformation in solvent-aqueous mixtures. Davis and Burns (1992) immobilized laccase on activated carbon, which enhanced phenolic effluent treatment. Another immobilized laccase displayed improved activity and stability in organic solvents (Ruiz et al, 2000).

5.3 Effects of solvent.

5.3.1 Other solvents.

Other solvents can be utilized in aiding the solubilization of PAHs, including methanol and dichloromethane. The stability of the laccase should be assessed in these and other solvents. Water-miscible solvents tend to vary in their hydrophobicity. The less hydrophobic a solvent, the more potential for it to be harmful to the enzyme by removing the necessary hydration sphere around the protein. Acetonitrile is a fairly hydrophilic solvent compared to others such as ethanol, acetone and tetrahydrofuran. It would be interesting to see the effects of these solvents on laccase and modified laccase. Perhaps the activity of laccase would improve due to the lesser ability of these solvents to replace the water in the hydration sphere. It must be remembered that the reason for addition of solvent to the system is to aid in the solubility of PAHs, so the solvent utilized must be able to solubilize PAHs and be able to mix with water without denaturing the enzyme.

5.3.2 Proportion of solvent.

It would also be important to determine the effects of the proportion of solvent present. Perhaps more solvent present will increase the amount of PAH

available for laccase oxidation. The chemically modified laccases were more stable in higher amounts of solvent than was the native protein and perhaps this will allow for a difference in oxidation rate. There is a limited proportion of solvent present in the experiments performed (15% acetonitrile was added to the reaction mixture). The true effects of solvent on kinetic parameters such as Km and Vmax and initial rates are evident at low water concentration, such as 10% water or less in organic solvent. The Vmax increased as water concentration increases but Km also increases. It would be of interest to test the enzymes under such conditions.

5.4 Other mediating substrates.

5.4.1 Synthetic mediating substrates.

Many compounds have been tested as mediators. In theory any good substrate of laccase could be used as a mediator. As ABTS and HBT are the most common mediators used they were tested in this study. However other mediators have been used and have been met with success. These mediators could be used in similar studies performed above to determine their effects on the degradation of PAHs by laccase from *C. gallica*.

5.4.2 Natural mediating substrates.

There have been several reports of natural compounds found in the culture supernatant that act as natural mediators. It is possible that this fungus also secretes a natural mediator. Studies using the ultrafiltrate from concentrating the supernatant by ultrafiltration could be performed to determine if there is an activity enhancing effect of the supernatant. This could be followed by the identification of mediating compounds in the ultrafiltrate.

5.5 *In vivo* metabolism.

A step towards practical use of laccase is to determine its ability to work in vivo in the context of bioremediation. Is the fungus able to work on a mixture of ^{14}C-PAHs in the presence of a lignin source? This may be tested using crude oil

that has a mixture of PAHs in the presence of a *C. gallica* culture testing for evolution of $^{14}CO_2$ or radiolabelled meetabolites.

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7. APPENDIX

7.1 Composition of lignin sources.

7.1.1 Oat Bran.

The only ingredient is oat bran and it does not contain any additives. Rogers Oat Bran has for a 35 g serving the following:

Protein	6.3 g
Fat	2.7 g
Carbohydrate	20.9 g
Fibre	5.6 g
Sodium	1.4 mg
Potassium	20.4 mg

Rogers Oat bran is produced by Rogers Foods Ltd. 4420 Larkin Rd., Armstrong, B.C., V0E 1B0.

7.1.2 Wheat Bran.

100 % Natural Bran contains wheat bran. It is distributed by Glencourt Distributors, Vancouver B.C, V6B 4E9.

7.1.3 Sawdust.

The sawdust was from pine wood.

7.1.4 No Name Bran Flakes.

The ingredients include the following: wheat flour, wheat bran, sugar, malt extract, salt, tocophenols, iron, niacin, thiamine, mononitrate, D-calcium pantothenate, pyridoxine hydrochloride, folic acid. No Name Bran Flakes has per 30 g serving the following:

Energy	119 cal / 500 kJ
Protein	3.5 g
Total fat	1.1 g

Polyunsaturates	0.5 g
Monounsaturates	0.1 g
Saturates	0.2 g
Total carbohydrate	24 g
Sugars	3.4 g
Starch	3.4 g
Dietary fibres	3.8 g
Sodium	224 mg
Potassium	157 mg

These bran flakes contain a variety of vitamins including: Vitamin B_1 and B_6, niacin, folacin and pantothenate iron. No Name ® product is prepared for Sunfresh Limited, Toronto, Canada, M4T 2S8.

7.1.5 Western Family Bran Flakes.

The ingredients include the following: whole wheat flour, wheat bran, sugar, corn syrup, malt extract, salt, ferrous fumarate, niacinamide, thiamine, mononitrate, calcium pantothenate, pyridoxine hydrochloride, folic acid. BHT was added to the packaging to preserve freshness. Western Family Bran Flakes has per 30 g serving the following:

Energy	100 cal / 420 kJ
Protein	2.9 g
Fat	1.1 g
Carbohydrate	24 g
Sugars	5.3 g
Starch	14 g
Dietary fibre	4.3 g

Sodium	265 mg
Potassium	160 mg

These bran flakes contain a variety of vitamins and other compounds including: Vitamin A, D, B_6 and B_{12}, thiamine, riboflavin, niacin, folacin, pantothenic acid, calcium, phosphorus, magnesium, iron, zinc. This product is manufactured by Western Family Foods, Vancouver, B.C., V6B 4E4.

7.1.6 Kellogg's All-Bran Bran Flakes.

The ingredients include the following: whole wheat, wheat bran, sugar/glucose-fructose, salt, malt (corn flour, malted barley), vitamins (thiamine hydrochloride, pyridoxine hydrochloride, folic acid, d-calcium pantothenate), minerals (iron, zinc oxide). Kellogg's all-bran bran flakes has per 30 g serving the following:

Energy	105 cal/440 kJ
Protein	3.1 g
Fat	0.7 g
Carbohydrate	27 g
Sugars	4.1 g
Starch	18 g
Dietary fibre	4.6 g
Sodium	260 mg
Potassium	160 mg

These bran flakes contain a variety of vitamins and other compounds including: vitamin A, D, B_1, B_2, B_6 and B_{12}, niacin, folacin, pantothenate, calcium phosphorus, magnesium, iron and zinc. This product is manufactured by Kellogg Canada Inc., Etobicoke, Ontario, Canada, M9W 5P2.

7.2 FPLC Protocol.

7.2.1 High Q anion exchange column.

A High Q cartridge (BIO-RAD) is a strongly basic anion exchanger with a $-N^{+}(CH_3)_3$ functional group. It has a bed volume of 5 ml with the binding capacity of ≥ 170 mg BSA. The recommended flow rate is between 0.5 – 3.0 ml/min and and pH range from 1-13. The recommended buffer is tris pH 7.5-8.0.

7.2.1.1 Phosphate buffer, pH 6.

Buffer A is 20 mM phosphate buffer, pH 6. Buffer B is 1 M NaCl, 20 mM phosphate buffer, pH 6. 5 mg of protein was loaded onto the column using an FPLC system (Pharmacia fine chemicals). The program to run the column is as follows.

Table 13. Program for columns using the FPLC system.

% of Buffer B	Time (min)	Flow rate (ml/min)
0	0-4	0.8
0-30	4-64	0.8
30-100	64-68	0.8
100-0	68-70	0.8
0	70-80	2.0

7.2.1.2 Tris buffer.

Buffer A is 20 mM Tris buffer, pH 8. Buffer B is 1 M NaCl, 20 mM Tris buffer, pH 8. 5 mg of protein was loaded onto the column using an FPLC system. The program to run the column was the same as above.

7.2.2 Mono Q anion exchange column.

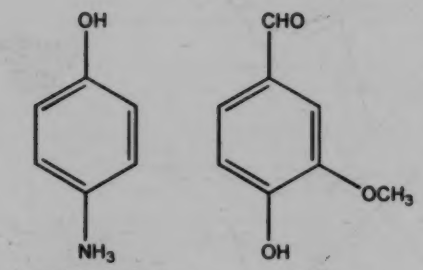
A Mono Q cartridge (Amersham Pharmacia) is a strongly basic anion exchanger with the charged group $-CH_2-N^{+}(CH_3)_3$. It has a bed volume of 5 ml

with a binding capacity of 0.28-0.36 nmol/ml of BSA. It is functional between pH 2-12.

7.2.2.1 Phosphate buffer.

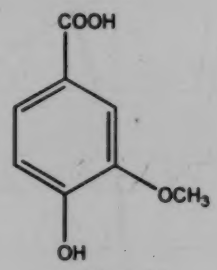
Buffer A is 20 mM phosphate buffer, pH 6. Buffer B is 1 mM NaCl, 20 mM phosphate buffer, pH 6. 5 mg of protein was loaded onto the column using an FPLC system. The program run on the column was the same as above.

7.3 Structures of known laccase substrates.

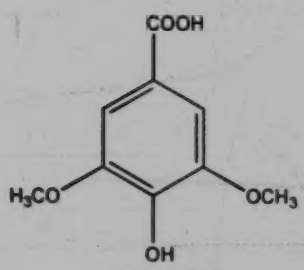


4-aminophenol

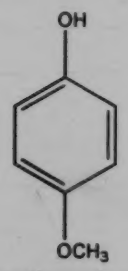
vanillin



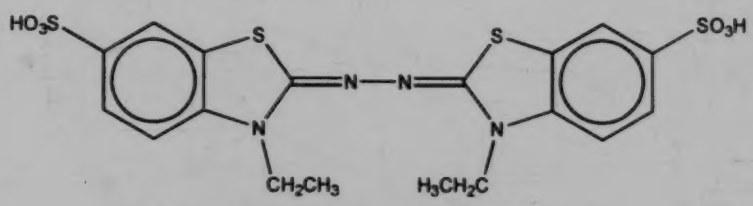
vanillic acid



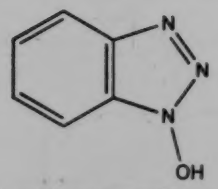
syringic acid



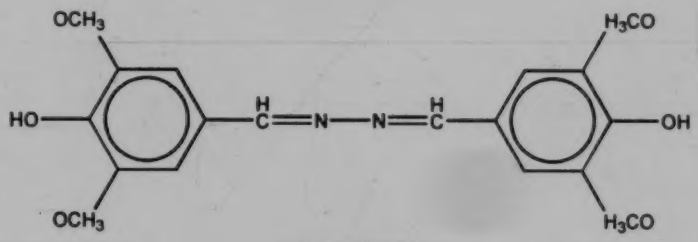
p-methoxyphenol



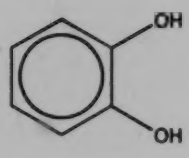
2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate)



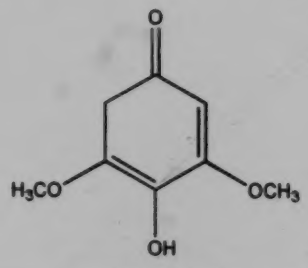
1-hydroxybenzotriazole



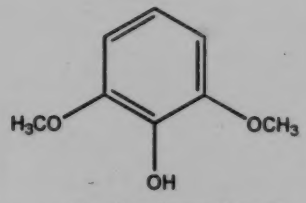
syringaldazine



catechol



syringaldehyde



2,6-dimethoxyphenol

7.4 Calculation of kinetic constants for laccase substrates.

7.4.1 Lineweaver-Burke plot.

Plot of 1/v vs 1/s

x-intercept = 1/Km

y-intercept = 1/Vmax

slope = Km/Vmax

7.4.2 Eadie-Holfstee plot.

Plot of v vs v/s

x-intercept = Vmax/Km

y-intercept = Vmax

slope = -Km

7.4.3 Hanes plot.

Plot of s/v vs s

x-intercept = -Km

y-intecept = Km/Kmax

slope = 1/Vmax

Values from each of the plots were averaged to determine Km and Vmax.

Vmax was then used to calculate Kcat using the molecular weight 56 KDa.

7.5 Initial velocity calculations for PAHs.

The data were fitted to the first order rate constant of the equation

$$A_t = A_o e^{-kt}$$

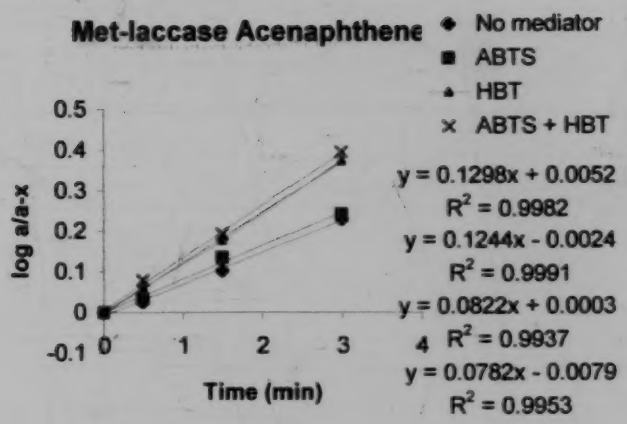
Average of three peak areas of triplicate runs

Calculate log A_o/A_t

A_o = initial amount of PAH

A_t = amount of PAH remaining

Plot log A_o/A_t vs t t = time



From the plot: slope = k / 2.303

$\therefore$ k = slope x 2.303

7.6 Sources of chemicals used.

All chemicals used in this study are alphabetically listed along with the company from which they were obtained.

Chemicals	**Source**
Acenaphthene, 97%	Aldrich (Milwaukee, W)
ACS Fluor	Amersham
Acetone	BDH (Poole, England)
Acetonitrile, HPLC grade	Fisher
Anthracene, 99+%	Sigma Chemical Co. (St. Louis, MI)
Anthraquinone, 99+%	Fluka (Ronkonkoma, NY)
L-Arginine	Sigma

2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)	Diagnostic Chemical (Charlottetown, PEI)
Bacto Potato dextrose agar	Difco Laboratories (Detroit, MI)
Bacto Yeast extract	Difco Laboratories
Benzene	Fisher Scientific Co.
Bio-rad protein assay dye reagent	Bio-Rad Laboratories (Hercules, CA)
Boron trifluoride-methanol complex	Aldrich
Carbo-Sorb II	Packard Instruments (Downer's Grove, IL)
Catechol, 99+%	Aldrich
Chloroform	BDH
Chrysene, 98%	Aldrich
L-Cysteine	Sigma
o-Dianisidine, 97%	Aldrich
Diethyl ether	BDH
2,6-Dimethoxyphenol	Aldrich
N, N-Dimethylformamide	Anachemia Canada Inc.
Dipotassium hydrogen orthophospohate	BDH
Dithiothreitol	Sigma
EDTA	Aldrich
Glucose	BDH Inc. (Toronto, ON)
1-Hydroxybenzotriazole	Sigma
L-Lysine	Sigma
Magnesium sulphate, heptahydrate	BDH
2-Mercaptoethanol	BDH
Methanol, spectral grade	Caledon
p-Methoxyphenol	Sigma
Methoxypolyethylene glycol activated with cyanuric chloride	Sigma
2-Methylanthracene, 97%	Aldrich
9-Methylanthracene, 98%	Aldrich

Naphthalene, 99+%	Sigma
Phenanthrene, 99+%	Aldrich
Phenanthrenequinone, 99+%	Aldrich
1,10-Phenanthroline monohydrochloride	Aldrich
Phenol (liquefied)	Fisher
Potassium dihydrogen orthophosphate	BDH
Pyrene, 99%	Aldrich
Sodium acetate	Caledon Laboratories Ltd
Sodium azide	Fisher
Sodium bicarbonate	Fisher
Sodium borate	Sigma
Sodium chloride	BDH
Sodium dodecyl sulfate	Boehringer
Syringaldehyde	Sigma
Syringaldazine	Sigma
Tris (hydroxymethyl) aminomethane	Boehringer
Urea	BDH Inc
Vanillic acid	Sigma
Vanillin	Sigma